MOLECULAR CHARACTERIZATION OF RED BLOOD CELL VARIANTS AMONG BLOOD DONORS AT THE NATIONAL TESTING LABORATORY NAIROBI –KENYA BLOOD TRANSFUSION SERVICE

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DECLARATION AND APPROVAL

Declaration and Approval

This study is my idea plus design and have not been presented anywhere else for a similar award.

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This work is devoted to my family, all altruistic blood donors, and recipients of blood transfusion, prescribers and those who collect blood from the donors.

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ABSTRACT

A genetic variant is an alternative nucleotide located at a specific region within a gene. To date, 48 genes encoding human red cell blood systems have been recognised. Variants within these genes encode for alleles, which can be highly polymorphic. Red cell nucleotides exhibit diversity among different populations worldwide. In Africa, there is limited information on the distribution and population frequency of red cell variants. In Kenya, there lacks red cell variant and blood group allele data to support with the investigations of alloimmunisation and management of rare blood types. This research therefore, pursues to generate molecular information of selected red cell variants in donated blood samples at the Kenya National Blood Service testing Laboratory. The rationale of the study was to generate molecular data on ABO, RH, MNS, Dantu, Kell, Kidd, Duffy red cell variants and predicated phenotypes. Also to contribute towards construction of a biobank (gene base/gene bank repository). The study design was experimental. Blood collected from donors for routine testing at the National Testing laboratory Nairobi of National Blood Service -Kenya was utilised. Next generation sequencing for molecular characterization of red cell variants was carried out using a custom panel on an Illumina MiSeq platform. Descriptive statistics were used in data analysis and results displayed in tabular formats. The results; ABO, O: 52%, B: 14%, A1:12%, $A_{2:}$ 5.6%, Ax 4.6%, $B_{3:}$ 4.6%, weak $A_{2:}$2.3% and the rest A1B or A_2B were below <1%. *RHD* gene: 30% genotyped as D+ 26% as D-, 10.2% as weak D, 4.6% as Del, with a number of other weak variants reported including 1% DAU6. *RHCE* gene:28% were c+e, C+V+VS+c+e+hrB+(17.6%),C+c+e+(14%),V+VS+c+e+hrB+(13%),C+V+VS+c+hrB+(11.1 %),JAL+Partial_c/VS+^{W}hrB+^{W}′-hrS+^{W}′ (6.5%).MNS system: 40% genotyped as N+s+, M+M^{c}+N+ s+(36.1%),M+s+(21.3%) and M+M^{c}+N +S+s+ (2.8%). KEL system: 76.9% genotyped as k+, (KEL2) Js^{a} (KEL6), Jsb (KEL7) at 15.7%, K+k+ (22%). Kidd system genotyping predicted 28% were Jk (a+b-), 24.1% Jk (a+b+), 22.2% Jk (a+b-), 17.6% as Jk (a+^{W}b+), 6.5% as Jk (a-b+),with 1.9% as Jk (a+ ^{W}b-). In the Duffy system, the null phenotype Fy (a-b-) genotype was the most common with ~90% of samples observed with this genotype. There was limitation in the characterization of Dantu variants because there are three different variants that are associated with Dantu and is not yet determined which one is in Kenya; thus, this will require more data and research to establish a reference gene to align them. A number of novel non-synonymous variants were identified in the dataset, which may be of potential immunologic significance in blood groups systems such as KEL and Augustine. This study has revealed a distribution of red cell variants for randomly selected Kenyan donors, in addition, valuable knowledge is presented in relation to rare and unusual variants as a basis for future research in Kenya. The study provides tools for extended typing in blood banks as a basis for improved transfusion practices and possibly for development of a genomic reference library.

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FUTI	Fucosyltransferase 1 (enzyme)
FUT2	Fucosyltransferase 2 (enzyme)
GDP-L	Glucose - Guanosine diphosphate fucose
GTA	A Glycosyltransferase)
GTB	B-Glucosyltransferase
UDP	Uni-Parental Disomy (existence of a gene pair resulting from single parent present in a disomic cell)
RBCs	Red Blood Cells
ISBT	International Society of Blood Transfusion
Bp	Base Pair
Aa	Amino Acid
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
Db	Data base
KNBTS	Kenya National Blood Transfusion Service
UDP	GalNac Uridine diphosphate N-acetylgalactosamine
UDP -Gal	Uridine diphosphate galactose
NACOSTI	National Commission for Science, Technology and Innovation

CHAPTER ONE

INTRODUCTION

1.1 Study Background

Red cell variants are alternative inherited nucleotide polymorphic (modified) forms of blood group molecules. They have been described as some of the earliest human Mendelian characteristics to be discovered. They form the genetic base of all the described human blood group systems. These variants exhibit distribution diversity worldwide. In African population, they are either absent or present in different forms. To date, 43 Blood Group Systems have been described. They are encoded by one or several genes. DNA encoding red cell surface molecules have now been cloned and have revealed that variations in genetic coding is within 48 different genes. These genes encode molecules range from carbohydrate, glycoproteins and proteins among others (Flower, 2017; Johnsen, 2015; Storry, *et al.*, 2016).

The blood group genetic location displays different types of inherited variants to include single nucleotide variants(SNVs), insertions/deletions, and structural variants(SV)/ organisational variations as displayed in the red cell gene database listing 1,600 alleles of 45 genes (Suneeti Sapatnekar, 2015). The International Society of Blood Transfusion (ISBT) review, curate all validated variants within the described 43 blood group systems. However, there are limited studies in the African populations resulting to underrepresentation in the International Data Bases. Information regarding these variants (genetic alleles) depicts the major importance of facts on blood types genes towards application of blood inventory management plus transfusion therapy. Identification of red cell variants can be accomplished by DNA sequencing via several techniques such as next generation sequencing (Kutner, *et al.*, 2014; Storry, *et al*., 2011; Tilley & Grimsley, 2014).

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Blood Groups Roles and Functions: Since the discovery of the ABO blood group system in 1900 by Karl Landsteiner in Australia, blood transfusion from human to human become possible and much safer. This science has continued to grow and to date 43 recognized blood group systems comprising 349 erythrocytes antigens genetically determined by 48 genes have been listed in the International Society of Blood Transfusion (ISBT) Website https://www.isbtweb.org/isbt-working-parties/rcibgt.html

Blood groups are molecules that are organised on the erythrocytes (red blood cells) surface membranes. These antigens are either polypeptides, glycolipids or carbohydrate that are the constitute portions of the erythrocytes cover and also allow communication with the immune system and give rise to phenotypes. The phenotypes are the noticeable personalities in an individual resulting from the countenance (expressions) of genes. Blood group categorization has been used in determining the human phenotypes. In addition, they have placed the human race in eight phenotypes to include A-Positive, B-Positive, A-Negative, B-Negative, AB-Positive, AB-Negative, O-Positive, and O-Negative; they also play other major roles such; carriage of some biological molecules in the direction of the red blood cell sheath, autologous complement regulators, enzymes, external stimuli receptors, anchors that connect the erythrocyte membrane to the cell skeleton, extracellular carbohydrates that shield the cell from mechanical and microbial attacks, and cell adhesion, structural components, components of the glycocalyx among others as shown in table 1 (Mitra *et al.*, 2014; Tombak & Tombak, 2019).

Table 1: Showing Blood Groups Functions & Medical Importance (Mitra *et al.*, 2014)

System	Protein Products	Role	Medical Importance
Diego(DI)	Band 3 glycoprotein-Anion Exchanger 1 (AE1).	Transportation of Anions	Development Barrier &Distal Renal Tubular Acidosis
MNS	GPA &GPB	Assists sheath assemblage of AEI	They serve as receptors for cytokines and pathogens, including the Plasmodium falciparum.
Rhesus(Rh)	D and CE polypeptides	Supports AEI/RhAG assemblage	Increases cations penetrability
Rh-associated glycoprotein (RhAG)	Rhesus linked membrane-bound molecules	Transporter of Noble or stable gases	Raise cations absorbency
Gerbich (GE)	GPC &GPD	Preserves erythrocytes oval shape by linking with 4.1 polypeptide	Raise red cell functions by permitting its form to interchange in the capillaries delivering oxygen and nutrients to other tissues
Cotton(CO)	membrane channel (AQP-1) Water Channel Protein	Water transporter	Transcellular water transport inside various organs.
Duffy(FY)	Glycoprotein(DARC)	Chemokine receptor for proinflammatory cytokines –Binding site of Malaria	Receptor for chemicals secreted by blood cells during inflammation and malaria infection,
KEL(Kell)	Glycoprotein	Zinc proteinase	an endothelin-3-converting enzyme; it cleaves "big" endothelin-3 to produce an active form that is a potent vasoconstrictor
Kidd(Jk)	Urea Transporter	Transports Urea	Maintain the osmotic stability of red blood cells
CROM (Cromer)	DAF/ CD56	Inhibits C 3 convertase	Microbes Recognition Receptor on Human Natural Killer Cells
Luthern(LU)	Peptide for laminin bound molecule	Promotes cell adhesion in the connective tissues	Facilitate intracellular signalling.
(XK)	Kell blood group precursor X-Linked VPS13A Binding Protein	Nucleotide carrier	(McLeod Syndrome)
Knops Blood	Complement receptor 1 (CR1)	key regulator of complement cascade activation, namely through accelerating the decay of C3 and C5 convertases	Reduce production of anaphylotoxins C3a and C5a9

The presence of naturally occurring antibodies is associated to exposure to antigens that may be abundant in the environment or may be produced as a consequence of active immunization to non-self-red blood cells immunogens via transfusions, pregnancy or transplantation. Presence or absence of inherited variation on the red cell surface antigens dictates the type of blood group a person carries. Blood group systems definition is based on one or more antigens governed by a solitary gene or combination of two or more closely related similar genes.

https://www.isbtweb.org/isbt-working-parties/rcibgt.html

The frequency of the blood group phenotypes shows variation among persons and humanities, and within the human tissues. Some of them are established just at the red blood cells whereas other are on both erythrocytes and tissues.

Medical Indications of Blood Therapy in Sub-Saharan Africa: The medical requirements for blood therapy entails a wide range of medical conditions to include types of anemia associated with sickle cell ailment, malaria, bleeding disorders, obstetrics such as anemia in gestation and obstetric bleeding, oncology, distress, critical operation, open heart surgery, burns and traffic road accidents. The key first approach to prevent and minimise blood transfusion related alloimmunisation is via an understanding of the cause or issues related to the medical condition (anaemia and coagulopathy) warranting a transfusion and adherence to documented evidence based guidelines on blood management However, in many Sub-Saharan African (SSA) nations, main blood requirements is wherever there is a crisis such as distress, anaemia-associated cases to include; malaria, SCD, and post-partum bleedings. There lacks or there are few documented guidelines on blood management in clinical settings in Sub Saharan Africa nations. Not much has been documented on how to monitor blood transfusion alloimmunisation both pre and post transfusion. In most of cases in Sub-Saharan African countries, pre-transfusion involves serological typing of ABO/D antigens and a four phase cross match. This method limits the antigen identification considering the other blood systems

that have been documented to be implicated in alloimmunisation. There is limited evidence or documentation on extended phenotyping for other clinical significant antigens such as Fy^b, MNS, Kell, Kidd, and RhCE and also a post transfusion look back to establish if the blood recipients were immunised (Barro *et al*., 2018; McGann et al., 2017; Chou et al., 2020).

Red Blood Cells Transfusion Associated Risk: Blood transfusion improves and saves lives. However, there exists a great risk of immunisation with immunoglobulins against the erythrocytes antigens in patients requiring repeated transfusions in cases of donor and patient antigen incompatibility. This includes sickle cell patients, in pregnancy and post-partum haemorrhage (PPH) after delivery, those undergoing kidney dialysis, malaria and oncology related cases. Serology is the current method of choice for donor and patient cross-matching in most developing countries including SSA. However, there is minimal or no extended screening carried out to assess the level of alloimmunisation/isoimmunisation. Despite the use of these methods (serological), the degree and complications of clinically significant blood group genetic variation still poses a major challenge in transfusion, pregnancy and solid organ transplantation (Boateng, *et al*., 2019). Today, most molecular basis of red cell gene variation has been revealed. With modern advances in molecular genotyping, (DNA sequence based blood typing) it is now possible to determine the red blood cell variants. This has significantly reduced cases of adverse transfusion reactions and transplant rejection (Johnsen, 2015; Manfroi & Pagliaro, 2014; Mindy Goldman, 2016; Sandler, *et al*., 2016).

Single Nucleotide Polymorphism (SNP) is a genomic modification at a certain site in the gene. It is the most common variants associated with the red cell genome. A SNP is valuable for revealing minor variations along entire genomes. The human genome contains more than ten million SNPs including those associated with red cell variants. Considering the many SNPs associated with the human genes, molecular genotype is preferred in red cell due to its many applications (Bugert, *et al*., 2008; Schoeman, *et al.*, 2017, 2018; Voelkerding, *et al*., 2010)

Immunological Implications of Red Cell Phenotypes-Alloimmunization (Isoimmunization): Alloimmunization/Isoimmunization is an immunological reaction by a person immune system to unfamiliar (foreign) human alloantigen mainly associated with pregnancy, blood components of transfusion and transplantation (antigens). In cases of immunological responses, foreign molecules (proteins, carbohydrate) anchors a specific antigens on their surface membrane that are responsible for antibodies production as an immune response towards the unfamiliar phenotype. Among the described 43 blood group systems, there are some phenotypes that are of clinical important due to their association with alloimmunization resulting to adverse transfusion reactions (acute & delayed), HDFN, haemolytic anaemia, as well as organ rejections. The main blood phenotypes that have been implicated in isoimmunization include; ABO, D, RHCE, MNS, Kell, Kidd and Duffy. In expectant mothers, isoimmunization occurs when the foetus and mother have different Rh phenotypes and this results to a condition known as HDFN where the erythrocytes are destroyed triggering anaemia and, in serious conditions it can cause foetus demise. Seldomly, fetomaternal isoimmune associated with thrombocytopenia (FMAIT) might be observed resulting to bleeding (Tormey & Hendrickson, 2019).

Red cell immunization due to transfusions are associated with acute adverse reactions in case of phenotype mismatch. In blood components recipients, isoimmunization occurs when the transfusion is from a different blood phenotype or sub-types thus the immune system marks it as foreign and mounts an immunological response by forming alloantibodies against the present alloantigen. It is also observed in recipients of massive /continuing blood therapy such as in cases of sickle cell disease, oncology, plus dialysis patients. These patients' immune systems are capable of developing alloantibodies plus others due to ongoing transfusions(Tormey & Hendrickson, 2019).

Most of the clinical important antigens mirror a single nucleic acid (amino acid) change differences between donors and recipients such as K/k+ while other significant antigens (such

as Rhesus system) reveal several nucleic acids (amino acids) alterations. These nucleic changes might be existing in the donors and completely absent in recipients (or vice versa). Other phenotypes are encrypted by multifaceted allele's (variants) that might be the triggers of an immunological response in relation to the formation of alloantibodies against the alloantigen**.** Transfusions, pregnancy or a transplantation involving a different blood type of subtype is the basis for the formation of isoantibodies (alloantibodies) in the recipient (Tormey & Hendrickson, 2019).

Red cells isoantibodies may be of clinical importance in future blood therapy (transfusion) or in other clinical conditions such as expectant mothers (pregnancy) among others. These agglutinins can result to severe acute or late haemolytic adverse events or HDFN in cases of mismatch between the mother and the foetus she is carrying. They might also result to prolonged and expensive appraisals in the blood bank and hamper identification of well-matched packed red cells and other blood components or products for subsequent infusions/transfusion. Due to the limitation of red cell isoantibodies identification, only small number has been recognised whereas most of them remain undetected. This has resulted to limited documentation on the morbidity and mortality burden associated with red cells isoimmunization(Tormey & Hendrickson, 2019).

Notwithstanding reducing transfusion related adverse events, nevertheless, isoantibodies (alloantibodies) development to infused blood products continues to be of medical significance in pregnancy, transfusion and transplantation therapy (Tormey & Hendrickson,2019).

Erythrocytes isoimmunization in sickle cell disease: Sickle cell disorder is an inherited red cells disease that is associated with anaemia. It is regulated by a one pair of genes. It is very common in the sub –Saharan Africa. Packed blood cells transfusion therapy is used to increase the oxygen-carrying capability to correct tissue hypoxia, anemia, reduce the manufacture of sickle young red cells and to avert problems associated with vaso-occlusive crisis and red cells

destruction(haemolysis). Continuous transfusion is associated to formation of antibodies or alloimmunisation/isoimmunization (Boateng, *et al*., 2019).

Erythrocytes isoimmunization/alloimmunization can cause delayed or stop a blood transfusion or complicate pregnancies (such as HDFN) resulting to increase the risks of future hemolytic transfusion reactions. Also patients who suffer from isoimmunization/alloimmunized have high risks of forming more alloantibodies and autoantibodies (Boateng, *et al*., 2019).

The major indications of packed red cell therapy in sickle cell recipients in Ghana include; reduced levels haemoglobin due to erythrocytes destruction and (haemolysis and malaria) and vaso-occlusive crises. There is limited continuous transfusion clinical guidelines and programs to be applied during the management of sickle cell multiple transfusions. The routine serological method in use prior to transfusion only types for ABO and D antigens and a compatibility test to compare blood types of the donor and recipient. There is no program or guidelines in relation to antibodies screening thus it is not one of the routine. Subsequently, the incidence of many red cell antigens not related to ABO-D isoimmunization/alloimmunization in patients receiving continuous transfusion therapy is unknown in most of the African countries including Ghana (Boateng, *et al*., 2019).

Considering the complications related to red cells immunization/isoimmunization in sickle cell patients, there is an urgent need to carry out antibody screen test to include extended typing for other clinical significance antigens beyond ABOD such as those related to RHCE, MNS, Kell, Kidd and Duffy. Genotyping is also important to aid in matching of antigen negative units for those who are continuously receiving ongoing transfusion therapy. To aid in the changes and addition of these pre and post transfusion tests, clinical transfusion guidelines and programs are required to be in place as reference documents in aiding the implementation of the new tests in regard to transfusion therapy (Boateng, *et al*., 2019).

Red cell isoimmunization/alloimmunisation in Pregnancy: Alloimmunization or isoimmunization might happen in expectant mothers (pregnancy) when the women and their

foetuses harbour different blood phenotypes (blood groups). ABO alloimmunisation in pregnancy is experienced when a mother does not have A and B antigenic structure on their erythrocytes whereas the fatal erythrocytes have the specific antigens. In most cases this is attributed to the fetus having inherited a different blood group from their paternal parent (father). This type of isoimmunization is not associated with major complications (Webb & Delaney, 2018).

Alloimmunisation with the Rh systems is common in mothers who are rhesus negative carrying a rhesus positive foetus. The exposure of the mother to the baby rhesus positive antigens may occur during pregnancy or during delivery. Once this occurs, the mother forms antibodies and in subsequent pregnancies, produced antibodies recognise the foetus as foreign and mounts an immunological response against the foetus red blood cells carrying the antigenic structure resulting to haemolysis. This may also result to an erythroblastosis foetal is due to the massive damage of the infant's erythrocytes. The degree of HDFN is dependent on the amount of antibodies present in the mothers' circulation). The Kell blood group antigens are linked with fetomaternal alloimmunisation thrombocytopenia (FMAIT) where the mother immunological responses are directed again the foetus platelets resulting to their destruction (Webb & Delaney, 2018).

The diagnosis of expectant mothers for alloimmunisation, an antibody screen test is conducted to establish if she has may have formed any immunoglobulins against the foetus' antigens. Furthermore, ultrasounds might aid to establish illnesses linked with isoimmunization through gestation, such as hydrops fetalis. After delivery, new-borns born of moms with established isoimmunization are evaluated for signs and indications of anaemia, screenings might be carried out to correctly identify the responsible immunoglobulins (Webb & Delaney, 2018).

Red cell alloimmunization in liver transplantation: Transfusion therapy is one of the vital treatment used for liver transplantation management. Isoimmunization to erythrocytes antigens is a major complication arising from transfusion therapy and in liver, kidney

transplants treatment which further becomes a future challenge. Patients expecting a liver transplant are likely to be managed with transfusion therapy and are consequently, prone to alloimmunization. If this isoimmunization is not identified and addressed, it might result late haemolytic transfusion reactions increasing the bilirubin levels that affect the liver functions and also complication in finding matched blood units. The Rh, MNS, Duffy, Kell and Kidd blood groups have been implicated in alloimmunisation in solid organ transplantation. Alloantibodies might also affect liver transplant consequences resulting to higher rates of early death (Makroo *et al*., 2017).

Consequently, testing for the presence of alloimmunisation via antibodies screening together with a balanced blood transfusion policy is vital in the management of these recipients. Erythrocytes isoimmunization may bring about an unusual challenge to solid-organ transplantation. Thus there is need for prior serologic testing of the recipient's pre-transplant as well as pre-transfusion to facilitate successful transfusion and solid organ management of these patients(Makroo *et al.,* 2017).

Determination of Red blood cells phenotypes: Pre transfusion determination of the antigens present in donors and recipients is very vital in reducing alloimmunisation. This procedure of identifying the type of antigens in child bearing mothers prior or during pregnancy is important as well as in pre-transplantation of solid organs. There are a number of techniques that can be applied in the determining the type of antigens that are present on the red cell surface membranes to include serological and molecular methods. Some of these methods that are currently in use include those that are done prior to transfusion and others are carried out in cases of complications related to pregnancies, adverse transfusion reactions and transplant rejection. The chief rationale of performing pre-transfusion red blood cell, phenotyping is a technique that is useful in offering the well-matched blood to the recipient in order to reduce the danger alloimmunization which can results to a haemolytic transfusion reaction. Typing and screening are the initial twofold investigations essential as a preparation for a transfusion.

The aim of typing is to determine the ABO/D antigens in both donors and recipients while screening test is carried out to identify the existence of antibody (ies) that may attack the red blood cells antigen(s). Antibody/antigen lattice formation is temperature dependent and also type of immunoglobulins present. Most antibodies that mount an immunological response against RBC antigens react best at both body temperature (warm) and cold (below body temperature) temperatures.

Erythrocytes Phenotyping: Red blood cells phenotyping is a serological procedure employed in the determination of specific antigens existing on the erythrocytes surface membranes. Identification of the presence of ABO and D phenotypes is carried out on all recipients in readiness for a blood transfusion. It is also carried out on donors who to determine the presence of ABO/D antigens. However, an extended antigen phenotyping might also be done to identify others antigens beyond ABO/D. This defines the antigen countenance other than the A, B or D phenotypes. Extended erythrocytes phenotyping is meant to complement the routine pre-transfusion testing in recipients with or suspected to have clinically significant isoantibodies (ies) in recipients suspected to be in danger of producing medically important isoantibodies(ies) such as those requiring ongoing transfusions (with sickle cell anaemia, oncology and post-partum haemorrhage) (Anural Mitra & Sarah Barnhard ; 2020.)

Red Blood Cells Extended Phenotyping: Erythrocytes/Rbcs antigen phenotyping is a serological technique performed using specific antibodies to identify detectable antigens anchored on the erythrocytes cell surface. This results to the determination of the revelation of an individual phenotypes that is dictated by the antigens demonstrated on the external sheath of the Erythrocytes/Rbcs. Presence of red cell phenotypes although not all the time generally links to the genes carried by an individual. Uses of phenotyping in blood banks are applicable for both donors and patients to include; confirm compatibility for a patient with an isoantibodies, identify donors with rare phenotypes**,** define which antigens the patient is carrying to prevent future antibody alloimmunisation and to confirm if the patient is negative

for an antigen to which he has become immunized (especially in cases of alloimmunisation with rare antigens such as those of Kell, Duffy and RhCE), the patient is phenotyped for these three antigens; anti- Fy^a, anti-e, K using known antibodies against a red cell panel (Anural Mitra & Sarah Barnhard ; 2020).

Red blood cells extended phenotyping panels: A red cell Panel cells should be composed of the cells of not less eight group O donors, at least two positive cells for each clinically significant and two cells negative for those antigens. It should also at contain least: one R1R1 cell (CDe/CDe), R1wR1 cell (CDe/CwDe) also expressing the antigens K, k, Fya, Fyb, Jka, Jkb, S and s between them, include one R2R2 cell (CdE/CdE), One r'r cell (Cde/cde), One r"r cell (cdE/cde), Three rr cells (cde/cde), At least one cell must be K+. Jointly, at least one cell must be homozygous for k, Fya, Fyb, Jka, Jkb, S and s. This will make this panel robust and useful in supporting the resolution of most antibody mixtures as possible (Anural Mitra & Sarah Barnhard ; 2020).

Types of red blood cells extended phenotyping panels: Types of red cell extended phenotyping is grouped into four major groups depending on the performance temperatures to include; 'cold phenotype'; 'full warm phenotype'; 'complete phenotype' and 'limited phenotype'. Cold phenotype' is a panel that governs manifestation of totally immunogens sharing same cold-reacting immunoglobulins (M, N, P, Lea and Leb). Cold reacting antibodies bind antigens strongly at lower temperatures (4^oc). The 'full warm phenotype 'identifies the phenotypes associated with most common medical important similar immunoglobulins that react best 37^oc to include; Kell, RhE, e, C, c, Fya, Fyb, Jka, Jkb, S and s). These types of immunoglobulins bind antigens strongly at 37^oc, thus the name warm reacting antibodies. The 'complete phenotype' is applied in the determination of all phenotypes which react best under cold and warm temperatures such as Kell, RhE e, C, c, Fya, Fyb, Jka, Jkb, S and s or cold reacting types to include: M, N, P, Lea and Leb. Some specific cases will warrant the use a 'limited phenotype' panel carried out to determine the presence of one or a few specific

antigens. It is worth noting that red cell phenotyping should be performed using a pre-transfusion specimen to reduce interference from transfused erythrocytes (Anural Mitra & Sarah Barnhard ; 2020).

Clinical indication for full warm antigen phenotyping: Full Warm phenotyping is useful in reducing red cell alloimmunisation in patients who are getting continuing transfusions and remain unprotected from to numerous alien RBC immunogens frequently over a prolonged period increasing the likelihoods of producing new isoantibodies. Consequently, performance of a complete warm phenotyping before transfusions permits the provision of fully or partially phenotyped-matched blood for these recipients reducing the chances of alloantibody (ies) production. The medical prescription given for a complete warm erythrocytes phenotyping prior to transfusions entails the following; freshly identified sickle cell disease cases, recipients with sickle cell disease who have not undergone a full warm phenotyping test, Extra haemoglobin disorders that require continuous or ongoing transfusion, to avert extra RBC immunoglobulins production – recipients of red cells who are already immunised face bigger danger of forming additional alloantibodies once exposed to new immunogenic antigens. Performance of RBC antigen phenotyping subsequently after finding isoantibodies is dire to offer best corresponding transfusions and prevent additional antibodies from forming. This is can be accomplished by performing complete warm phenotyping or restricted phenotyping building on the impulse testing pathway, in preparation for medicines known to hamper testing such as in patients being treated with anti-CD47 (such as Hu5F9) which is linked to interfering with the red blood cell antibody screen. A complete warm phenotyping procedure ought to be is carried out preceding drug administration to permit phenotype matched transfusions throughout the medicine administration in case the fresh immunoglobulins are not detectable consequently allowing safer transfusions (Anural Mitra & Sarah Barnhard ; 2020).

Clinical indication for complete antigen phenotype: Phenotyping the patient's erythrocytes immunogens consistent with specific immunoglobulins that react best in cold temperatures is

normally carried out when the recipient has produced a cold- reacting immunoglobulin. Examples of such case are those observed with M antobdies that occur naturally and are common in young children or antibodies to Lewis that is frequently linked with pregnancy) (Anural Mitra & Sarah Barnhard ; 2020).

Clinical indication for a restricted/Limited antigen phenotype: A limited or partial phenotyping details describes a single or numerous certain erythrocytes immunogens which can be applied as an alternative in place of a complete phenotyping technique. In most of case, it is performed to: determine antibody specificity (immunisation in patients lacking immunogen). In case an antibody pool detects a single or numerous isoantibodies of undistinguishable specificity, carrying out a limited antigen phenotype might be of assistance in determining the actual specificity associated with that antibody (ies), identify those patients who have developed an immunoglobulin, the laboratory is required to carry out phenotyping for the matching antigen as well as others that are known to be more immunogenic, evaluate threat associated with hemolytic disease of the new-born. Fatherly or cord blood RBC antigen typing defines the antigen countenance that matches to the maternal antibody, occasionally carried out post transfusion to assess erythrocytes recovery is as per expected. Defining the recipients individual erythrocytes phenotype after-transfusion to establish number of circulating red blood cells in comparison to the pre-transfusion antigen typing can support in defining the rate of RBC recovery (Anural Mitra & Sarah Barnhard ; 2020).

Restrictions to RBC Phenotyping: Red blood cell antigen phenotyping has some limitations in its application; cases of a Positive Direct Immunoglobulin Test: (DAT+) could be a sign of an autoimmune hemolytic anemia (AIHA), recipient's erythrocytes are covered with IgG or IgM immunoglobulins resulting to a false positive phenotype testing, and consequently the antigen profiling cannot be consistently defined, recent Blood Transfusion: In cases of a recent transfusion: the phenotyping is usually performed using the patient's red blood cells and due to recent associated transfusion, there will be presence of circulating donor red cells in the

midst of recipients erythrocytes .Consequently, extended phenotype testing results will not be reflecting the recipients normal RBCs. The medical profile of transfusion is vital in such cases, some medications: for those undergoing treatment that might be coating the RBCs example of drug that might coat the RBCs resulting to interference (anti-CD47). If the patient's RBCs are covered with medicine, and consequently the antigen profiling cannot be dependably defined (Anural Mitra & Sarah Barnhard ; 2020). .

Clinical implications of extended antigen: In recipients requiring comprehensive antigen matching, the antigen summary of every donated units which have been infused has to correspond with the NEGATIVE antigens in the recipient antigen profile adding to any antibodies. If the recipient does not receive frequent transfusions and single or numerous agglutinins/immunoglobulins are identified in the initial period, transfusion facilities ought to carry out an extended antigen typing especially for the immunogenic antigens and offer packed red blood cells. This in turn will reduce chances developing a hemolytic transfusion reaction and development of fresh immunoglobulins. In case the recipient attention requires a lifetime continuing transfusions, a complete warm RBC phenotyping is carried out before all transfusions. In certain times defining a negative antigen profile is challenging both to the transfusion services as well as to pathologists promoting need for a risk stratify the antigens by immunogenicity to find the finest existing match. This will be accomplished through carrying out the impulse testing in order to find a donor red blood cell units that are extended phenotyped and matched (Anural Mitra & Sarah Barnhard ; 2020).

Collection of Phenotyping Sample: Whole blood sample is placed in EDTA test tube. EDTA chelates calcium consequently acting as an anticoagulant allowing separation of plasma from red cell with centrifugation. The plasma separated from whole blood is used for antibody screening testing whereas the red blood cells are used for antigen phenotyping (Anural Mitra & Sarah Barnhard ; 2020). In General, the phenotype panels required necessitate testing timing allocation 1.5-2 hrs. Identification of some phenotype to specific antigens requires a prolonged

incubation period whereas others are defined quickly. Their identification are accomplished by use known commercially acquired antisera after incubation with the recipient's erythrocytes/Rbcs. Antihuman globulin reagent is **a** prerequisite when using monoclonal IgG associated antisera. This encourages agglutination and confirmation of the antigen present (Anural Mitra & Sarah Barnhard ; 2020)**.**

Prophylactic red cell antigen matching: Red cell antigen matching for Rh (C, E or C/c, E/e) and K antigens on addition to ABO/RhD matching for patients with SCD (all genotypes) receiving ongoing blood infusions due to suspected alloimmunisation. Red blood cells Matching reduces alloimmunisation and increases post transfusion red cell survival (Chou et al., 2020). Limiting adverse immunological responses in patient at risk of alloimmunisation

Haemolytic transfusion reactions are uncommon health complications mostly associated with alloimmunisation. They are life-threatening types of anaemia that necessitates instant red cell transfusion. However, at times it becomes difficulty to find compatible blood that is free from alloantibodies for whom antigen negative blood units is required. This necessitates provision of guideline documents suggesting immunosuppressive therapy (intravenous immunoglobulin, steroids, and/or rituximab).

Blood Transfusion exchange in isovolemic hemodilution for patients with SCD: Blood recipients are inducted on a red cell reduction procedure including simultaneous volume replacement (normal saline or 5% albumin). This is aimed at reducing the number of red cell pints required. A guideline panel is essential to guide in the red cell exchange detailing the red cell exchange procedure(Chou *et al*., 2020). Blood infusion management through pregnancy: standard requirements suggests both protective transfusion at systematic interludes or quality attention (transfusion when clinically directed for a problem or haemoglobin below the standard) for expectant mothers with SCD (all genotypes) (Chou *et al*., 2020)

Guideline target group: These guidelines will be of great use in helping blood prescribers or clinicians while making decisions on diagnostic and treatment choices. Additional resolutions

to inform policy, training, awareness and national prospective study needs. In additional, these may also be useful for recipients/patients. The recommendations are not formulated to be inferred as a standard of care. Health care givers will be required to come up with judgements based on the clinical appearance of separate single patient, preferably via a collective practise reflecting the patient's principles plus inclinations through reverence to the expected consequences of the selected choice. Pronouncements might be regulated via the practicalities of a definite clinical situation and confined means, plus but not restricted to established strategies, time restrictions, and accessibility of treatments. These guidelines do not necessary include all appropriate methods of care for the clinical scenarios described. As science develops and fresh proof is made accessible, references might become obsolete. Adherence to these guidelines is not an assurance of actual results. Announcements about the primary morals and inclinations, as well as succeeding interpretations associated with every recommendation, are its essential portions and assist to expedite correct clarification. They ought not to be forgotten while citing or interpreting references emanating from these guidelines. Application of the guidelines will be enabled via upcoming resolution supports (Chou *et al*., 2020). The guidelines will require support from the management, stakeholders a political good will. This will require advocacy to create awareness and acceptance by sharing this study outcome with the relevant stakeholders to include: ministry of health policy makers, clinicians (private and public via their unions, institutions of higher learning and other key players (Chou *et al*., 2020).

Blood management in pregnancy and childbirth: Patient blood management (PBM) has now become an integral of treatment care. It encompasses a series of events and approaches to ensure maintenance of an optimum haemoglobin (Hb) levels, improve equilibrium, reduce loss of blood, and maximum blood transfusions in the pursuit of improved patient outcomes. Current research show that usage of PBM reduces perioperative haemorrhage, decreases necessity of transfusions (Surbek et al., 2020). Nevertheless, several obstetricians and obstetrical branches still require direction on how to implement PBM in day-to-day medical

practice. Regardless of the confirmed benefits, challenges and mind-sets hinder the application of PBM guidelines every day while giving health care services. These encounters can be due to lack of advocacy, cross-practical commitment, adequate resources, and miss conception of PBM profits might even engender one job for those working in blood donor service or transfusion medicine. Initiating PBM in transfusing health facility or hospital necessitates the inclusion of practicing physicians plus extra health care workers who consider transfusions as an initial medication in to correcting low haemoglobin levels. PBM is founded on three pillars to include: anticipation, detection, and correction of preoperative/prepartum anaemia; two; prevention and reduction of perioperative/peripartum loss; and three enhancing postoperative/postpartum management of anaemia which entails regulating usage of erythrocytes infusions. Perhaps, the most difficult assignment is to persuade hospital management teams leaders of the significance of PBM and to change their stand of health management amid their colleagues(Surbek *et al*., 2020).

Hemodynamic transformation during pregnancy: Throughout gravidity, a variety of physiological changes occur in the hemodynamic, cardiovascular, and coagulation-fibrinolysis systems take place. These changes are associated nature in ensuring reduced blood loss throughout pregnancy to delivery. In the first trimester, there is an upsurge in blood volume .The high volumes of blood makes a rapid expansion during the 2nd trimester (30–50%) reaching a stabilized level in the last 3 months of pregnancy. In contrast, the amount of RBC increases nonetheless to a lesser extent (20%), resulting to a comparative reduced blood levels (anemia) owing to dilutional; reaching its highest level 30–32 weeks of pregnancy. Diluted haemoglobin is regarded as a normal physiological development in pregnancy, particularly in the middle weeks 28 and 34, during which haemoglobin levels drop to the lowermost During the initial period of the pregnancy, the erythrocyte mass surges to approximately 18–25%, which is reduced during and after delivery owing to peripartum hemorrhage .The Increase in erythrocyte mass ensures sufficient oxygen for the amplified need from both mum and fetus.

These physiological changes have significant profits through pregnancy: The placenta has a healthier perfusion, the danger of coagulation drops, sufficient blood quantity is guaranteed regardless of hemorrhage occurring during childbirth. Uterine artery blood movement surges through pregnancy (10 times) and reaches 450-750 ml/min at the end of the gestation period (pregnancy) term In comparable, there is a considerable rise in clotting ability with an upsurge of the clotting factors I (fibrinogen), VII, VIII, IX, X, XII and von Willebrand factor. Moreover, there is a reduction of F XIII and a physiologic decline of protein S, whereas F II, V plus protein S remain unchanged. The raise of plasminogen activator inhibitors 1 and 2 reduces fibrinolytic action. Consequently, there is an upsurge of the thromboembolic danger (Surbek et al., 2020). In summary, hemodynamic and hemostatic vicissitudes signify adjustments of nature to the challenges of reproduction and are fundamentals for a fruitful pregnancy product of the mom and the baby. However, post-partum hemorrhage (PPH) remains a central reason of maternal illness and mortality during delivery (Surbek *et al.*, 2020).

Factors associated with postpartum haemorrhage: The major factors associated with severe PPH consist of uterine atony, retained placenta, placenta praevia, placenta accreta, and placental abruption, trauma involving uterine rupture, or lower genital tract trauma and primary coagulopathy. Explanations of PPH from a clinical viewpoint are listed as Trauma (of birth canal), Tissue (remaining placenta or placental pieces), Tone (decreased uterine muscular tone: atony), and Thrombin (coagulopathy). Women associated with earlier PPH during their previous gestation, pre-existing anemia, former caesarean section, many gestation, uterine fibroma, pose a greater danger for PPH among others. Nevertheless, PPH can happen in any expectant female at any age even without a prescribed risk or danger. Consequently, we have to anticipate that all expectant women are always in danger of PPH. (Surbek *et al.*, 2020).

Iron deficiency and anaemia in pregnancy: Preoperative low hemoglobin level is normally disregarded in operation. The invasive/ operating technique is regularly executed per scheduled, and blood is issued when regarded as an essential part of the standard care. In

obstetrics, there is an exceptional chance to identify iron insufficiency and low hemoglobin level long before a probable blood loss, as well as in each of the subsequent appointments of gestation care. Consequently, ideal basics are present in the implementation of the initial step of PBM, before including the optimization of the red blood cell mass at delivery. Overall, low hemoglobin levels are frequently associated with expectant pregnant women and is generally linked with iron insufficiency. Additional causative factors are related to haemoglobinopathies, thalassemia, and sickle cell anemia, infections such as hook worm, malaria, Vitamin B_{12} insufficiency, or protracted inflammation. In 2011, the proportion of anemic expectant mums worldwide was at 38%, showing widespread variation in different regions globally. In the Middle East Region, 48.7% of the expectant women had anemia, in Africa 46.3%, while in Europe 25.8%. If the factors associated with pregnancy anemia are identified and resolved at the earliest time during the gestation period, it would enhance healthy benefits of the mother and the baby. This would also ensure the mother will not receive blood transfusion due to PPH which in turn would reduce red cell antigens alloimmunisation(Surbek *et al*., 2020).

Blood group genotyping Technology: The replicating and sequencing of blood group genes has made it easy in the identification of nucleotide variations accountable for blood group mutations (polymorphisms). One of the major technology that made blood genotyping possible in the non-specialist molecular genetics laboratories was polymerase chain reaction (PCR). PCR amplification enabled analyses of the DNA sequencing of a small region of a blood group gene, using a small quantity of total genomic. After the PCR application, many methods such as next generation sequencing are now being used in red cell genotyping (Daniels, 2021).

Haemagglutination (Serologic) and molecular genotyping: Correct erythrocytes typing of both donors and recipients patients is essential in the prevention of alloimmunization and haemolytic transfusion reactions. Serology red cells antigen identification is significant in the quest of well-matched units for transfusion therapy. Molecular genotyping has been employed blood transfusion therapy in order to supplement the haemagglutination test investigation and

documentation of the definite phenotype or the existence of allemorphs that must be associated with causing cause adverse transfusion reactions compromising transfusion security. Nevertheless, it is critical for one to remember that a genotype is only the inference of a phenotype, particularly when associated with the existence of a mute genes and nonexistence of genetic material augmentation (Menegati, e*t a*l., 2020).

Blood Group Molecular Genotyping and Applications: Blood group genotyping also known as molecular genotyping is a technique applied in the predication of blood group phenotypes from genomic DNA sequencing. It is also referred as molecular blood grouping as well as genomic identification (testing).Many blood group mutation are linked tom one nucleotide mutation. Identification of the nucleotide status in relation to zygosity (homozygous or heterozygous) at the location of the SNP will regularly predict present phenotypes with a high level of precision. The accuracy of molecular genotyping to correctly predict present phenotypes is dependent on the genotyping platform and the expertise of the users (Daniels, 2021). There are various reason when a phenotype need to be known. However of the many reasons, three are critical to include; lack of a suitable red cell sample, to provide a better reliable phenotype data compared to serological testing, plus when the molecular typing is further efficient and cost effective in comparison with the serology test. In other scenarios incorrect outcomes may arise between phenotyping and genotyping due to gene sequence alterations separating from the SNP being determined affect the antigen manifestation such as in the determination JK (Kidd) genotype where the antithetical Jka (JK1) and Jkb (JK2) result from c.838G>A in SLC14A1 (Daniels, 2021).

Blood genotyping may also be used in the prediction of antigens that are expressed weakly by serological techniques such as very weak Fyb (FY2) antigen also denoted as Fyx which is not detected by serological techniques. While the highest number of genotyping tests encompass determining variation in the genes coding the antigen (e.g., Rh, Kell, Duffy, and Kidd systems), others include identifying variation in genes coding glycosyltransferases (enzymes)

accountable for the biosynthesis of sugar antigens [e.g., ABO (20)] or of regulator arrangements governing the gene expression (Daniels, 2021).

Benefits of Molecular Genotyping: Blood group molecular typing is associated with and enormous range of applications in transfusion medicine, obstetrics, and transplantation medicine to include**:** defining RhCE variants; their identification is key when finding a suitable donors for sickle cell disease patients to reduce antibody production (These variants c,e are common in the people of African origin); screening apparent D– donors for the presence RHD, this is critical in the confirming they do not have a weak variant such as DEL antigen , which is regularly missed by serological methods but then again it is capable of causing alloimmunisation in D– patient or boost a pre-existing weak anti-D; preimplantation genetic diagnosis can be used for avoidance of HDFN when a blood group antibody, which has already caused severe or fatal HDFN, is present in a woman whose partner is heterozygous for the allele encoding the offending antigen.

This is common to the D phenotype is homozygosity for a deletion of RHD; defining whether a D+ person has one or two copies of RHD (i.e., hemizygous or homozygous), Zygosity testing is potentially valuable for testing fathers of foetuses at risk from HDFN When non-invasive foetal testing is available, this test is seldom necessary. Genotyping can replace serological tests that are unreliable or when suitable antisera are unavailable for rare phenotypes such as FY, and KEL among others of clinical significance; also useful in the serological reference laboratory in helping to solve complicated cases especially in the documentation of uncommon antibodies, exome sequencing (by next-generation sequencing).

Blood group genotyping on patients who have recently been transfused whose red cells are covered with immunoglobulin in vivo (DAT+); determination of D (RH1) variants and of RhCE variants in patients; screening apparent D– donors for weak expression of D Testing patients for multiple clinically significant blood groups and their variants Blood grouping when serological reagents are rare or unreliable, preimplantation genetic diagnosis for avoidance of

HDFN Assistance with identification of blood group antibodies in the reference laboratory Screening donors for multiple clinically significant blood groups and their variants Determination of RHD zygosity ABO typing from buccal swabs in transplantation registries A1/A2 typing in solid organ donors and defining foetal blood group to evaluate danger of HDFN Determination of foetal blood group to appraises necessity for anti-D immunoglobulin (Daniels, 2021).

Quality Control, Assurance and EQA in Blood Group Genotyping: Quality control and assurance is an important component of quality system essentials (QSEs) that supports a laboratory to quality program at the same time guiding a dynamic involvement in EQA. Quality Assurance involves all measures that can be taken to improve Blood bank's effectiveness and efficiency with a vision to the improve safety and benefit to the persons receiving the service. External Quality Assurance goals is to ensure that the data generated by a laboratory are consistent , accurate and dependable, thus improving trust and performance of Blood Banking and Immuno-hepatology services offered by Blood Banks. Continual upgrading of blood bank laboratories performance can be accomplished by performing internal quality control and participating in external proficiency scheme. Due to improvement of medical laboratory science automation of equipment as well as the regulation of blood banks testing protocols, the accuracy and quality of blood bank has seen a radical positive change globally (Sippert et al., 2019; Daniels, 2021). Quality assurance in transfusion medicine comprises the usage of and involvement in internal and external quality programs. Quality management is vital to guarantee that laboratory performance is consistent and precise on a day-to-day basis. Nevertheless, an external quality assessment scheme (EQAS) that matches results from different laboratories is crucial to validate the accurateness and dependability of laboratory results. (Sippert et al., 2019; Daniels, 2021). The main aim of participating in EQA is to measure, uphold and improve as required by the standards of performance in laboratories. Support for the national acceptance of EQA can be recognised by making advocating for a

criterion for upgrading, signifying the profits of top practice, and offering evidence, training and funding for improvement(Sippert et al., 2019; Daniels, 2021)

Quality Control in Molecular Genotyping: The use of molecular typing for the description of blood group genes has been gradually growing in the last few decades due to the transfusion benefits for the recipients, technological developments in molecular practises, and intensifying accessibility of mass-scale genotyping. Whereas blood group genotyping is attracting more and more use, typing errors have been acknowledged demonstrating importance of quality control. When blood group genotyping is being applied for medical reasons, it is essential that it is appropriately controlled. This regulation should take in consideration participation in an EQA programme (Sippert et al., 2019; Daniels, 2021)

Rationale and Benefits of EQA: EQA is very effective in rising the standards of a blood bank laboratory when the prerequisite for quality is acknowledged. This must be augmented by guarantee support of senior management to back the developments chosen to advance performance. Involvement in EQA is considered as an effective means of championing standard performance in situations wherever quality systems are missing. EQA outcomes can uncover poor performance and promote in recognizing the necessity for standards, guidelines, education and training, and the essential resources needed to sustenance the standard of a blood bank laboratory(Sippert et al., 2019). The profits of an EQA program to health and regulatory authorities entails the following; network of blood transfusion laboratories encouraging standard of performance; training and education of laboratory staff and Provide valuable evidence to support in: setting standards; testing strategies reviews, test kits, reagents and instruments post market survey, effective use of resources, improved public confidence in the provision of blood transfusion service and backup structures of accreditation (Sippert et al., 2019) . The advance and accessibility of reference reagents will enable improved quality control in assay production, assessment of performance and proficiency of testing by specialized laboratories making patient care stress-free and safer to offer (Sippert et al., 2019).

Guidelines for red cell transfusion in Sickle Cell Disease: The evidence generated form this study is useful in guiding the development or reviewing existing framework to assist health worker and patients in making decision in relation to transfusion provision for SCD and the managing of transfusion-related complications. Red cell transfusions continue to be the main therapy for patients with sickle cell disease (SCD). However, it still carries substantial medical issues. Alloimmunization, delayed hemolytic transfusion reactions (DHTRs), and iron overload screening, prevention, and management, as well as guidance for specific transfusion indications and administration, may help improve the outcomes. These guidelines should include all areas that should be seen as the source of alloimmunisation to include: Red cell antigen profiling panel suggests an extended red cell antigen profile by genotype or serology beyond ABO/RhD typing should be performed as early as possible for all patients with SCD (all genotypes) both pre and post transfusion. The extended panel should include all the blood group phenotypes mostly associated with alloimmunisation (ABO, D, C, E Kell, Kidd and Duffy). The composition of an extended red cell antigen profile is composed of C/c, E/e, K, Jka/Jkb, Fya/Fyb, M/N, and S/s at a minimum. The Red cell antigen profiles need to be provide across hospital systems blood banks and blood service. . A serologic phenotype may be inaccurate if the patient has been transfused in the last 3 months. Blood group genotyping is more over serologic phenotyping, as it offers extra antigen information and delivers increased accuracy for, amongst other things, C antigen determination and Fyb antigen matching (Chou et al., 2020)

1.2: Statement of the Problem

Every 10 minutes, seven people require a blood transfusion in Kenya. Blood transfusion, and transplantation therapies improve and save life. However, they are never without danger. This is because they are associated with red blood cell alloimmunisation in recipients and organ rejection. This is mostly associated with the blood donor and transfused patients red cell genetic variability together with innate /adaptive immune factors. Complications associated with RBC

alloimmunization include; late transfusion due to the investigations of new alloantibodies, challenges in identifying compatible blood for highly alloimmunized individuals, and delayed haemolytic or serologic reactions and organ and tissues rejection.

Currently Kenya is applying serological methods to carry cross matching of donor and recipients in the identification alloantibodies for both as the pre and post transfusion alloimmunisation investigations. However, Serological method is limited in the full detection of alloantibodies thus presenting missed detection opportunities especially in selected recipients and immunized pregnant women of child bearing age.

Non availability of variant and blood group allele data has contributed to the challenges associated with the investigations of alloimmunisation, donor and recipient antigen matching plus management of rare blood types.

1.3: Justification

Molecular genotyping is a key method in investigating the occurrence of RBC variants/ alleles in the populace, supporting matching of red cell blood units and also as the first step in building a red cell variant database to guide transfusion safety. The generated evidence from this study will be useful in decision makers in terms of blood management policy reviews and development, testing protocols and clinical guidelines on ongoing transfusions in relation to red cell alloimmunisation. It is also useful inform on the development of a red cell gene Bank in Kenya. This study is very timely to address the existing gap and also of importance for the ISBT.

1.4.: Study Significance

This study outcome is envisioned to inform the requirements for developing a blood group genotyping reference laboratory in Kenya and also for the East African region. A molecular genotyping reference laboratory is fundamental in the investigations of complicated immunohematology, transplants as well as matching of donors and patient red cells,

particularly for those patients receiving multiple and ongoing transfusion therapy for example (sickle cell and dialysis patients).

1.5. Study Objectives

1.5.1: Broad Objective

To determine via molecular characterization the distribution of red blood cell variants (ABO, D, MNS including Dantu, Kell, Kidd and Duffy) in blood donor samples from the National Testing Laboratory Nairobi-Kenya of the National Blood Transfusion Service and develop a needs analysis of requirements for implementation.

1.5.2: Specific Objectives

1. To determine ABO genotype distribution in voluntary blood donors of Kenya applying molecular typing.
2. To determine D and weak D variants distribution in voluntary blood donors of Kenya applying molecular typing.
3. To establish the frequency of variants in the "minor" systems (c, e, MNS including Dantu, Kell, Kidd and Duffy) in voluntary blood donors of Kenya using molecular typing.
4. To determine the frequency of novel blood group variants amongst voluntary blood donors of Kenya derived from molecular typing.

1.5.3: Research questions

1. What is the ABO genotype distribution in voluntary blood donors of Kenya, derived by molecular typing?
2. What is the distribution of significant Weak D variants amongst voluntary blood donors of Kenya?

3. What is the frequency of distribution of red cell variants in the systems of "minor" blood group antigens (c, e, MNS including Dantu, Kell, Kidd and Duffy) in voluntary blood donors of Kenya?
4. What is the frequency of novel blood group variants amongst voluntary blood donors of Kenya derived from molecular typing.

1.6: Scope of the Study

This investigation seeks to generate a molecular characterization dataset of red cell variants in a randomly selected group of voluntary blood donors in Kenyan

1.7: Study Limitation

The available commercial serological antisera for grouping was limited in capturing most of the red cell variants.

1.8: Delimitation of the study

The focus will be on erythrocytes molecular variation among the altruistic blood donors in Kenya (African variants).

1.9: The Study Assumption

That the determined amount of blood specimens was illustrative of the blood donor population in Kenya. All reagents were viable and equipment working state acceptable and available; and required temperatures (room temperature) was maintained

1.10: Operational Definitions of Key Terms

Data Base	Organised exceptionally evidence data stored in a computer and is accessible using different methods
Gene bank	Genetic material preservation storage or a biorepository
Biorepository	A biological materials repository (store)

CHAPTER TWO:

LITERATURE REVIEW

2.0: Introduction

2.1.1: The ABO system

The ABO group pattern was the first to be reported in 1900 by Karl Landsteiner. This was during one of his experiment when he noted that some erythrocytes from different persons when mixed with serum from other individuals were clumping (agglutinating). He went ahead to demonstrate that this phenomena was associated with some specific makers on the surface of the erythrocytes. These surface markers have now become to be known as antigen A and B. The existence of either A or B antigens denotes the phenotype of each person. Those persons who showed presence of both A and B antigens on the erythrocytes surface membrane were added to the ABO system as AB blood type. The ABO antigens are used to determine the blood type of all humankind. An individuals' blood group either belongs to type A, B or AB. Erythrocytes that lack these markers (A, B and AB) are grouped or named as type O. The ABO blood group variants are differentiated through the existence or non-appearance of antigens on the erythrocyte membranes and immunoglobulins in the plasma (serum). ABO is among the most documented phenotype among all the 43 described Blood Group Systems (Dean, 2005; Hosseini-maaf, *et a*l., 2007).

The ABO variants are of clinical significance in transfusion, pregnancy, transplantation and immunology. This is because they have been implicated in immunological reactions in cases of a mismatch due to the presence of their antibodies in the blood. Thus, accurate determination of these variants prior to transfusion and transplantation is critical. In adverse complicated cases of transfusion and transplantation reactions and rejection, genomic typing has become useful in determining the involved red cell variant. These variants show variation among different races (Dean, 2005; Flegel, 2013; Goebel, *et al.*, 2013; Periyavan, *et al*., 2010; Storry, *et al.*, 2016).

2.1.2: ABO Nomenclature and Biochemistry

The discovery of ABO was a major breakthrough in transfusion medicine and placed it at number 001 in the list of ISBT. Classification for A variants has been clearly defined. The antigens position is determined by absence or presence of specific defined sugar structures, especially for A and B variants. AB and A_1 antigenic structures are not clearly defined compared with those of A and B (Dean, 2005; Flegel, 2013; Goebel, *et al.*, 2013; Periyavan, *et al*., 2010; Storry, *et al.*, 2016)**.**

2.1.3: Biochemistry of ABO

The ABO structures are made of carbohydrate molecules. They are also given the name histo blood group due to their presence in other body tissues (Hosseini-maaf *et al.*, 2007). They display genetic polymorphisms with three alternative variants A, B and O. Antigens A and B expression is codominant, however O is a recessive type. The formation of the ABO antigens requires a precursor molecule known as the H substance. This precursor component also defines the H antigen (Lalueza-Fox, *et al*., 2008).

The H antigenic structure is responsible for the configuration of ABO variants. The control of H antigenic molecule is under the influence of two genes; *FUT1and FUT2* located on chromosome 19. *FUT1* gene catalysis synthesising of H molecule on RBC (type 2 precursor); Whereas, *FUT2* is associated with presence of H antigens on type 1 precursor on secretions. These genes encode an enzyme known as fucosyltransferase (2-α-L-fucosyltransferase) which is accountable in conveying the donor sugar (L-fucose from guanosine diphosphate fucose (GDP-L-fucose)) to the end galactose ancestor types 2 (Hosseini-maaf *et al*., 2007). The ABO enzymes (gene products are known as GTA (A) and GTB (B). Each of them require UDP Gal substrates as shown in **table 2** (Hosseini-maaf, *et al*., 2007; Liehr, 2010)

Table 2: Showing internal structure of ABO blood types (Hossein-maaf, et al., 2007)

Precursor structure		Determinant		Location
Type 1 →	Galß →	3GlcNAcß1 →	R	e.g.endodermal cells
Type 2 →	Galß →	4GlcNAcß1 →	R	e.g. erythrocytes
Type 3 →	Galß →	3GlcNAcß1 →	R	e.g. O-linked
Type 4 →	Galß →	3GlcNAcß1 →	R	e.g. Glycolipids

R, indicates the carrier which can be carbohydrate, glycolipid or glycoprotein

2.1.4: The ABO Antigens and Enzymes (Glycosyltransferase)

The ABO blood group displays major antigens to include; A, B, O and AB. These immunogens are located on the surface membrane of the red cell molecules. These molecules are also found in other body tissues and secretions. They also have antibodies that occur naturally in plasma and serum. ABO (H) molecules (antigens) are located on type 1 and type 2 predecessors. Type I are the major transporters of ABH antigens in body fluids including plasma and serum; while the type 2 are the main receptors for ABO enzymes (glycosyltransferases) linked through the red blood cells membranes. Type 3, cyclic A- related sequences found arranged on the erythrocytes carbohydrates or else are produced by blood type A persons (Hosseini-maaf, *et al*., 2007). Group 4 chains are present in small quantities on erythrocytes and in elevated amounts in renal cells of individuals belonging to phenotype A. The type 4 ABH organizations are individually established on glycolipids (Hosseini-maaf, *et al*., 2007; Svensson, 2011; Yamamoto, *et al.*, 2017).

2.1.5: ABO Enzymes (Glycosyltransferase)

The ABO genetic structure is defined by two closely linked macromolecules; α- (1 → 3) – *N* – acetylglucosaminyltransferase (GTA) and α- (1→3)-galactosyltransferase (GTB) known as glycosyltransferase (Alfaro *et al.*, 2008). GTA and GTB exist as homologous glycosyltransferases with a minor variation in 4 of the 354 nucleotide (amino acid) (Arg/Gly176,Gly/Ser235, Leu/Met266, and Gly/Ala268) sequence in GTA and GTB correspondingly (Alfaro *et al.*, 2008). They are linked during the formation of the ABO (H) antigenic structures. GTA is responsible for the transportation of GalNAc after UDP-GalNAc to H antigenic receptor (α-L-Fuc-(1→2)-β-D-Gal-*O*-R in the production of the A antigen. GTB moves Gal after UDP-Gal towards the H antigenic receptor resulting to the formation of B antigens (Alfaro *et al.*, 2008). Blood group O lacks an active transferase while AB phenotype has both the GTA and GTB enzymes (glycosyltransferases). It has been shown that there is an association between ABH molecules during formation of ABO system as shown in **fig 1** (Denomme, *et al.*, 2009).

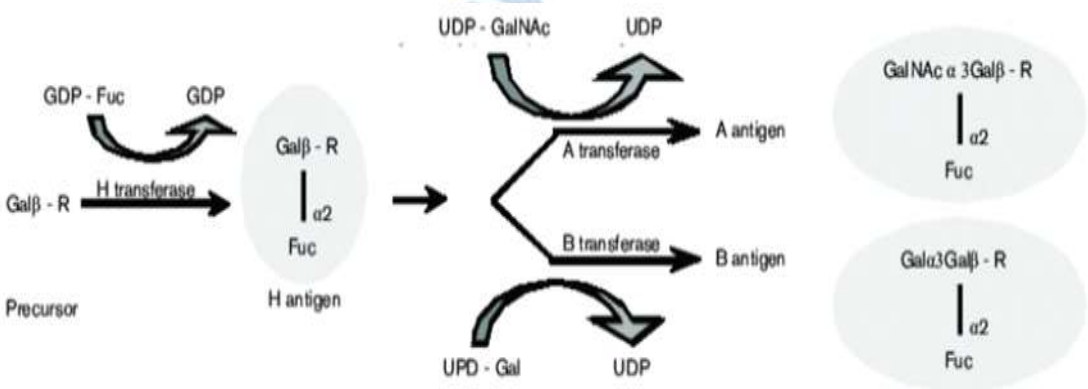

Figure 1: Schematic illustration of biosynthetic pathways for the conversion of H structure to A and B elements; R represents the core structure (Olsson, ML & Storry JR., 2009. ABO Blood group System revisited: A review and updated

2.1.6: ABO Weak Variants (Subgroups

In comparison to other described blood group structures, ABO displays a noticeable variation in the antigenic arrangements present on the erythrocyte surface membranes. In most cases, manifestation of ABO (H) antigens might be associated with various influences such as precursor type and quantity; expression location and disease. ABO antigen quantity is determined by the individual genotype. For others, the quantity of antigenic structures available may be defined by non-ABO heredities or ecological influences. Hereditary genetics that are resulting from ABO genes are known as subgroups, variants or weak forms of ABO. These weak variants are categorized depending on the amount of epitopes associated with A or B antigenic structures. In cases where the quantity of antigenic epitopes are few in comparison with principal phenotype (e.g. A1 or A2), they are classified as weak types. It has been demonstrated that in the A phenotypes, A1 has about five times the number of antigens than on RBCs compared with A2 (Denomme et al., 2009; Olsson, ML & Storry JR, 2009). The primary source for the quantifiable variations amongst them is not mostly associated with the activity of the transferase enzyme. The A2 enzyme transferase has been shown to be ten times less effective than the A1 type. The A2 transferase enzyme is limited in its capacity to work with other chains except type 1 or 2 carbohydrate precursors like the extended type 3 (repetitive A) and type 4 (globo-A) series on the erythrocytes carbohydrates structures (Denomme et al., 2009; Olsson, ML & Storry JR, 2009). To differentiate the ABO genetically recognized as weak groups and those determined by non ABO genes or ecological causes; those associated with gene are referred as weak subgroup while those linked to environmental are called weak phenotypes. Most research work have demonstrated a strong relationship between DNA arrangement variation existing in the encoding site of ABO gene and the character (A or B antigens) and amount (A2, A3, Am,Aw,Ax, Ael, B3, Bel,) of the phenotypes. This have been shown to arise from ABO gene mutations (Bugert et al., 2008; Denomme *et al*., 2009; Svensson, 2011;Hosseini-maaf, *et al*., 2007; Olsson, ML & Storry JR, 2009).

ABO system is also associated with a null phenotype known as Bombay type. The Bombay phenotype (Oh) individuals are known to display silenced FUT1 and FUT2 genes. Since the H antigenic structure is the ancestor assembly for A and B antigens, and thus none of them will be produced without α2FucT activity despite the presence of the ABO genes resulting to lack of both A and B immunogens on the external membrane of the individual red blood cells. Para-Bombay phenotypic expression is produced in two scenarios; either a muted FUT1 gene is existing in conjunction with an activated FUT2 gene, allowing production of H type 1 (thus A/B antigens) that might be assimilated on erythrocytes as of the plasma; or silenced FUT1 gene which the coded transferase action is significantly reduced, thus resulting to little quantity of H antigen (and A/B antigen) being formed. This might exist with or be devoid of any activity in FUT2 gene. In such circumstances the H antigen (A/B antigen) will be inadequately conveyed consequently becoming quite difficult to detect their presence using routine serological methods. In 2015, the Red Cell Genotyping 2015 symposium approved use of molecular genotyping to enhance precision of complicated red cells variants determination. This was to enhance patient clinical care and donor safety (Denomme *et al*., 2009; Svensson, 2011;Hosseini-maaf, *et al*., 2007; Olsson, ML & Storry JR, 2009).

Identification and distinguishing weak subgroups of "A" by serological assays is promising a great range with technical expertise; nevertheless, molecular genotyping is important since it confirms the result. Mistyping weak subgroups of A as "O" group results to reporting and documenting incorrect ABO blood phenotype in a blood donor, mis-grouped packed red cell components erroneously labelled as "O" and mistakenly transfused to a "O" group patient may result to an adverse transfusion event such as haemolytic transfusion reaction. A patient of weak A subgroup mistyped as "O" and transfused "O" whole blood might suffer an adverse transfuse reaction. In addition, unanticipated naturally occurring anti-A_1 might bring about ABO group discrepancy, differing with compatibility testing, and if not detected may result to a transfusion reaction thus is clinically significant (Thakral *et al*., 2005).

2.1.7: Molecular Genetics of *ABO* Variants/ Alleles

The ABO antigenic structures are inherited in line with the Mendelian traits in a codominant autosomal (non sex chromosome) style. The ABO antigenic structure was the first to be used toward the testing of fatherhood (parental) and criminology science (ML, J.S., 2009). The genetic location for a protein molecule polymorphism is simple and clear. In most cases, the difference is associated with a single nucleotide polymorphism (SNP) resulting to one amino acid modification. This amino acid alteration might expressly dictate the polypeptide molecule antigenicity especially if it linked to a blood group transporter. It is important to note that there could be various genetic loci that may underlie a blood type and this may result to different genetic variants from same phenotype (Hosseini-maaf *et al.,* 2007).

The ABO blood group gene is situated on the end part of the long arm of chromosome 9 (9q34). The location of the ABO gene has been known for many years prior to its cloning. This cloning was made possible in 1990 by Yamamoto and colleagues when they successfully isolated and purified nucleotide sequencing of A enzyme after lung tissue (ML, Olsson, and Story .J., 2009; Hosseini-Maaf *et al.,* 2007; F. Yamamoto, *et al*., 1990).

The ABO gene coding sites are composed of seven exons (1 exon has two variants) in the A1 –mRNA copy. The coding region is approximately 19.5 kb and ranges between 28-688 base pairs in size. Of the seven coding regions, 6 and 7 are the longest containing 823 of the 1062 base pairs (figure 2). The two exons (6 and 7) cover 77% coding polypeptide or 91% of the active part of the ABO enzymes (glycosyltransferase). Exons 1-5 determine the amino – terminal cytosolic (cytoplasmic) area, a membrane-spanning area (a.a.17-37), the stem section plus the residual 9% of the enzyme active area shown in **figure 2** (Hosseini-*maaf et al*., 2007; Rummel & Ellsworth, 2016).

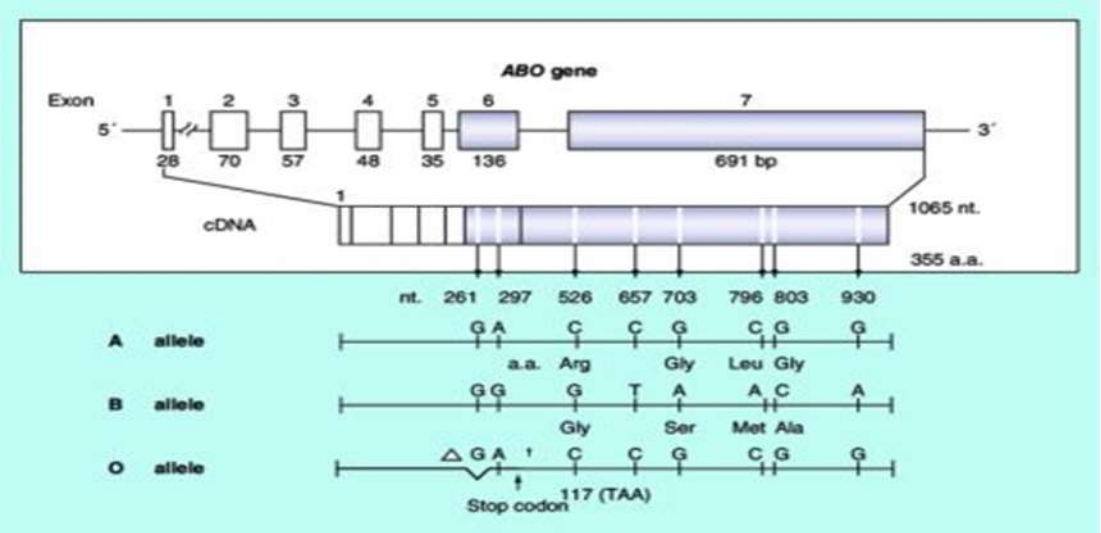

Figure 2: Showing ABO gene locus and exon organisation of the coding sequence (shaded) and nucleotide sequences of A, B, and O alleles. Total Changed a.a. sequence in O alleles due to frame-shifting caused by single base pair deletion. a.a. (Amino acid); bp (Base pair); nt (Nucleotide position) (Rummel & Ellsworth, 2016).

In-depth search of the Complementary DNA (cDNA) libraries for human cell lines linked to ABO phenotypes, the major alleles were defined. The Yamamoto team went ahead to show how the B mRNA vary from A specific gene by only 7 of the 1062 encoding amino acids. The resulting altered nucleotides, four are the products of nucleotides variation in the transferases products (F. Yamamoto *et al*., 1990).

The ABO genes have been shown to be extremely polymorphic in comparison with the other described blood group systems. It has to be noted that several main alterations are attributed to a number of ABO blood types. The difference between the A1 [A101] and O1 [Oo1] gene was found to be a single nucleotide (G) deletion. This substitution of a single nucleotide alters and adversely shortens the open reading frame (ORF). The A2 blood type (phenotype) is due to deletion of cytosine in the 5' stop of the gene causing in lengthening of the opening reading frame (ORF). From the time of the first paper of ABO cloning, approximately 215 sequence entries were already uploaded in the data base of erythrocytes (dbRBC) by 2009 (Hosseini-maaf *et al.,* 2007; ML, 2009; Svensson, 2011).

Due to the polymorphic nature of ABO genes, new alleles are continually recognized. Of the 215 alleles, 181 were grouped via their link with normal or mutated phenotypes. Of the 181 alleles, 65 are of various A alleles, 47 of group B, 58 fall in the O alleles, while 11 are of ‘’AB’’ types. The other 34 alleles in the data base for red blood cells (dbRBC) site have twenty arrangements surrounding the 5’ non coding site comprising the repeating and changing(heterogeneous) CCAAT box binding factor /nuclear factor Y(CBF/NF-Y) structure and 14 intronic or intersection arrangements (ML,2009). Out of the A allomorphs, 6 and 11 code normal A1 or A2 blood types(phenotypes) on the red blood cells respectively (ML,2009). The remaining 48 A alleles (variants) depict mutated (weak) forms (A weak) which is linked to various alleles of weak A antigen countenance (ML, 2009). The B alleles consist of nine typical and 38 weak allomorphs (variants/alleles) of which eleven of them have been shown to encode glycosyltransferases that are able to produce and demonstrable amounts of both A and B antigenic structures (antigens). These B allomorphs are capable of expressing two uncommon types’ cisAB and B (A). There are approximately 58 allomorphs (alleles) which are envisaged to produce polypeptides devoid of any enzyme action. Forty five of these produced proteins, have 261delG an alteration that changes the conversional ORF and bring about a truncated protein product devoid of enzyme (transferase) action (Hosseini-maaf *et al.,* 2007; ML, 2009; Svensson, 2011).

The resulting shortened protein products lacking the enzyme activities belong to the large phenotype of O alleles including four major lineages; O1 ([Oo1], O1v [Oo2], Otlseo9 or O1 (467T; 318T) [Oo9] and O1bantu [O54]. Various negligible variants of the major alleles, O hybrid alleles plus A2 or B including the altered 261delG with O arrangements have been established. This type of combination has been found to be common in persons of the African origin. Among the thirteen remaining “nondeletional’’ antigenic variants, 3 are associated with nonsense mutation which lead to changed open reading frame (ORF) which is due to nucleotide insertion. Three of the remaining alleles have nonsensical alteration initiating instant stop

codons resulting to a shortened ORF at the same codon location. The last seven remaining antigenic variants were shown to have nondeletional O alleles which are damaged by one missense modifications resulting to significant amino acid substitutions example 802G>A changing to 268Arg. Interestingly, most of the nondeletional O alleles are all associated to mutation in A antigenic variants (alleles) organisation sequences. This has led to two main practical significances; one being that nearly all ABO molecular typing techniques will indicate existence of A1 or A2 variants (alleles) except if they are mainly intended to identify rare O alleles. If care is not taken to ensure the genotyping procedure is specific to identify the rare O variants, this can result in blood type O person categorised as an A phenotype. The second significance is that the blood type that was inherited by these O alleles, are not all the time O but frequently weak A or O like that lacking anti A in plasma. No known O allele (Variant) has been based on a B sequence. (ML, 2009; Hosseini-maaf *et al*., 2007).

The molecular hereditary genetics of the ABO system is interesting due to the mutations and variation alternating from one amino acid alteration to additional complicated cross gene configurations capable of changing the specificity or effectiveness of transferase or both at the phenotype antigenic amount displayed by changed (altered) antigen countenance. An example of such changes are demonstrated by various molecular genotype characterisation of the Ax phenotype (weak form of group A1) resulting from only one nucleotide alteration in exon 7 of the A1 [A101] allomorph or occur due to various combination (hybrid) such as those among the 5' end of B and 3' end of the O allomorphs. Apart from the changes in the specificity and efficacy associated with the enzyme activities, unusual intracellular trafficking of ABO enzyme (transferase) may result to weak forms or subtypes groups (phenotypes). This extra intricacy in which mutation influence the enzyme activities without involving the antigen makes it difficult in devising genotyping techniques (methods/assays) used in detecting the rare variants. If this is not interpreted correctly, it can result to grave consequences in ABO phenotype identification (ML,2009)..

More than 30 ABO commercially screening genotyping assays are now available and in use. Most of these methods are only intended to identify about 3 and 6 of the frequent allemorphs; even though extra allemorphs that are unexpected occur in case of mutations making it difficult to identify them in regard to the limitation of the method in use. Due to the complexity and modification of the ABO, new alleles keep developing (ML, 2009). Thus there is need of using genotyping method in the developing countries to aid in ascertaining the existing variants (Alleles) and novel types that may be present in the ABO system. This will also be crucial in aiding the development of new screening genotyping methods and red cell antigenic panels that will be very supportive in resolving complicated cases associated with pregnancy, transfusion and transplantation (Storry & Olsson, 2009; Svensson, 2011).

2.1.8: ABO Phenotypes in Relationship to Diseases

Variances in blood group antigen countenance (expression) can rise or reduce host vulnerability to various diseases. Some blood groups are associated with disease predisposition while others are linked to their protection. The ABO blood group system is also known as the histo blood group system because it found in both red cell surface membranes as well as in most tissues. They are also carbohydrates molecules meaning heavy molecules with high or big molecular weight. Due to the factor that they carbohydrates associated antigens and that their antibodies are naturally present in plasma, ABO has been described as is the most immunogenic blood group system. The presence of ABO antibodies naturally occurring has been associated with boosting the innate immunity against some parasitic infections and at the same time enhancing others. Different blood groups have been shown to be associated with different infections due to their locations and functions (Mitra et al., 2014; Cooling, 2015; Ward et al., 2020, Samra et al., 2021) .

2.1.9: ABO Blood Group Phenotypes in association with COVID-19

Acquired adopted influences governing exposure to SARS-CoV-2 and cruelty of COVID-19 are yet to be completely understood. Some studies that have been carried on blood groups in

association to disease show that ABO might have some effect on the predisposition of COVID-19 and cruelty of the disease (Samra et al., 2021) . Samra and colleagues carried out a study on ABO blood phenotypes to establish if they are associated with dangers for the infection of SARS-CoV-2 consequential in COVID-19. They found that precisely phenotype A was linked to an increased threat in comparison to type O. The investigators also revealed that author's type O individuals were at lower risk of COVID-19 infections compared to non-O persons. The study also established that antibody A prevent binding of glycosylated SARS-COV S protein-conveying cells to angiotensin-converting enzyme 2 (ACE2) on cell external, consequently these immunoglobulins might musk the communication among the virus and its receptors thus giving protection. They also found that the action of Angiotensin-converting enzyme 2 (ACE2) in phenotype B is abundant compared to the amount present in type O leading to increased chances of being infected with the Covid-19 virus which explains the reason of high number of death was higher in B phenotype in comparison to type O (Samra et al., 2021)

Samra et al., 2021 findings also demonstrated that there was association among type A and rise in vulnerability to thromboembolism, diabetes mellitus, and hypertension, recurrent urinary tract infections from Escherichia coli, and Helicobacter pylori that is linked to ulcers, stomach cancer, and heart diseases. This association can be the explanation of the increased number of death mortality of COVID-19 in those persons categorised as A phenotype. The Samra team established that individuals of A phenotype have an increased chance of contracting COVID - 19 and developing severe symptoms compared to those of type O (Samra et al., 2021).

The roles and functions of ABO are linked to disease protection due to their naturally occurring antibodies and role in innate immunity. The ABO antigens are made of carbohydrates and thus linked to anchoring of other molecules and microbes thus becoming the entry of these harmful agents. Considering the association between ABO blood types and COVID-19 infections and

mortality, it might be helpful to introduce ABO blood typing in the management of COVID-19.

2.1.10: ABO Blood Group Phenotypes in association with Vibrio cholerae bacteria

Cholera is a serious diarrhoeal condition associated with the consumption of food or water polluted with the *Vibrio cholera* bacteria. It is one of the disease that is regarded by WHO as a worldwide risk to public health and also a pointer of inequality and deficiency of community progression. WHO has listed a number of fundamental evidences in relation to Cholera infections including;

- Greatest number of those with disease show no signs or if present they are not early detected and can be effectively managed with oral rehydration fluids.
- A universal approach on cholera control, Ending Cholera: a global roadmap to 2030, with a goal to decrease cholera mortality by 90% was initiated in 2017.
- Investigators have projected that each year there are 1.3 to 4.0 million cases of cholera, and 21 000 to 143 000 case mortality globally owing to cholera (1)
- Cholera is a serious diarrhoeal illness that can be fatal within hours if left unmanaged.
- Ensuring there is availability of clean drinking water and hygiene in all areas are pivotal in the prevention and controlling the transferring of cholera and other waterborne diseases.
- Serious cases will require swift management with intravenous solutions and antibiotics.
- Oral cholera vaccines should be used in combination with enhancements in water and hygiene to control cholera outbreaks and for stoppage in zones identified to be great danger for cholera https://www.who.int/news-room/fact-sheets/detail/cholera

In relation to ABO blood group association with Vibrio Cholera, a study carried out in 1977 by Barua and Paguio evaluated patients who were being treated for Cholera at the Pavilion, San Lazaro Hospital, and Manila. Among those who presented with diarrhoea infections, the highest percentage were found to be phenotype O (77%) against 45%, and lowest frequency

was found to associate with type A against 26% persons than expected. However, on subjection to culture they found that both O and A were giving the same outcome between those who were Vibrio-reactive and those who were non-reactive (Cooling, 2015). .A succeeding research established a twofold rise in the percentage of type O persons at 61.5% against 33%, complemented by a reduced fraction of phenotype B people reported as 14.5% against 35%, amongst those admitted Bengalese patients. To assess if there was correlation of the two studies, Levine and colleagues carried out an investigation to affirm the two outcome by recruiting sixty six men who offered themselves to be inoculated with Vibrio cholera. Though many of them (55/66; 83%) developed diarrhoea, group O phenotypes were double as probable to progress to serious disease 64% against 36% of those who were either A,B or AB phenotypes. They also reported from their study that type A was associated with a significant reduction in cholera vaccine effectiveness (Cooling, 2015).

Cholera toxin is known to bind weakly to the ABO antigens and this may be acting as decoys to draw the toxins away from its true target. Type O is devoid of the ABO antigens thus may not be a good decoy for the toxin leading to persons of this blood type being more severely ill with cholera than those of the other blood types. This is because cholera toxin hyperactivates a key signalling molecule cyclic adenosine monophosphate (cAMP) signalling in the intestinal Enteroids cells leading to excretion of electrolytes and watery diarrhoea (Kuhlmann, *et al.,* 2016).

2.1.11: ABO Blood Group Phenotypes in association with Alloimmunisation

ABO antigens are carbohydrate molecules on the surface of red blood cells. They have naturally occurring antibodies in the plasma and other body tissues. During transfusion, transplantation or pregnancy, they can be introduced to persons who lacks the specific antigens and thus will initiate an immunological process by forming antibodies against them. Thus the importance of ensuring they are well identified and the red cell units are matched to reduce alloimmunisation in the recipients.

2.1.12: ABO Genotyping

ABO genotyping is applied in many situations to include blood transfusion, transplantation; personal identification and diseases detection for instance erythroblastosis fetalis (haemolytic disease of the foetus and new born or HDFN). ABO antigenic structures include; A, B and AB and are located on the erythrocytes external membranes. They determine human phenotypes. The ABO system is regulated by a single or a group of genes located on the same chromosome 9 (Flegel, 2013). The ABO antigenic variants are inherited traits shared among humans and apes (Farhud & Yeganeh, 2013; Wayman, 2012).

2.3: RH Blood Group System

The Rhesus structure is second in medical importance behind ABO. It was described in 1940 by Karl Landsteiner and Weiner during their experiment with the rhesus monkey which led to the discovery of a genetic association between the monkey antigen and that found on the human enterocytes surface membranes. The antigens were identified by immunoglobulins/agglutinins produced in bunnies and later sensitized with rhesus monkey erythrocytes. This association of the two antigens resulted to the D molecule incorrectly being named as the Rhesus antigen. An evidence of Rhesus continues to be applied up today in the blood group system Rh that comprises D and RhCE antigens (Daniels 2013). The Rh system is composed of three major antigenic structures (molecules), D, C, and E that are non- glycoside/ transferrin (phospholipid) polypeptide. It has been shown that the distribution of the D, C and E epitopes (antigens) varies among races. D+ phenotype is approximated to be around 85 % in the Caucasian, 95 % among the Africans and greater than 99.5% in the region of East Asia. This system is also associated with subtypes to include weak forms to include; weak form of D, partial D, and DEL. All these names have now been reviewed and revised to a single term known as the D variants (Daniels, 2013). The system is also associated with a null type that is devoid of any antigenic molecules. The RHCE is also of clinical importance in terms of immunisation especially the little e and c. Their distribution among different races show variations with the e type being the most

common at 98.42%, C at 87.55%, c: 51.06% and lowest being observed in the big E at 26.55%, c-51.06%. These variations are dependent in specific populations (Sarkar *et al*., 2013).

2.3.1: The molecular genetics of Rh and D polymorphism

The Rh system organisation comprise of twofold genes namely; *RHD* and *RHCE* positioned on gene 1p36.1. They are closely related sharing 93.8 % similar introns plus encrypting exons. Though interrelated/interlinked; their location is positioned on opposite alignment in the chromosome with ten coding regions encrypting 417 nucleotides as shown in **fig 3** (Daniels, 2013). The RhD and RhCE proteins differ from each other with 31 to 35 of amino acids. They span the red blood cells membrane 12 times. The RhD and RhCE protein molecules are commonly found in most populations (Daniels, 2013). The Rh D negative type is due to total removal of the D genetic material. Amongst white race, the D– characteristic is linked with whole removal of the D gene. Among black Africans, approximately 67 % D– express a replica of a quasi-gene of RHD*Ψ (pseudogene) associated with deactivated 37bp repetition in exon 4 substitution (Tyr>stop) and an alteration occurring in exon 6 (Floch, A. (2021); Daniels, 2013; Rizzo *et al*., 2012).

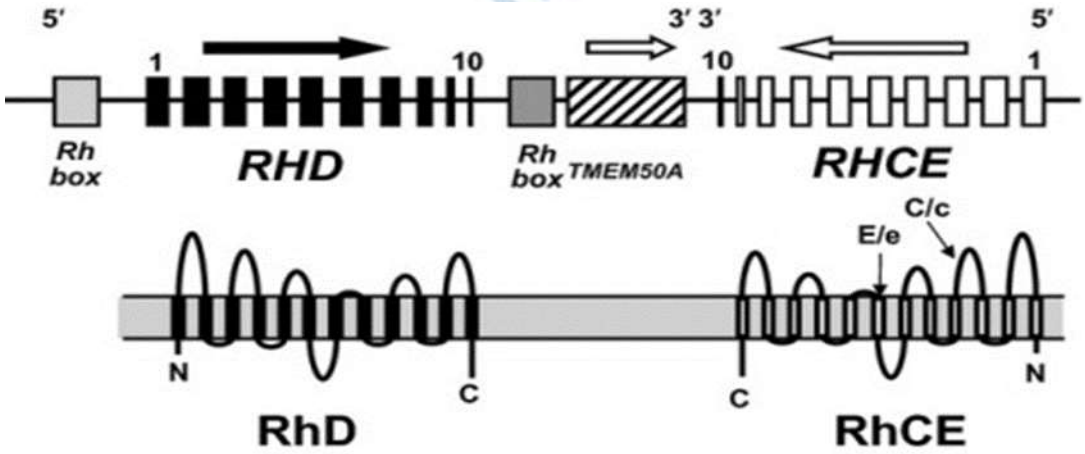

Figure 3: Shows the organisation of RHD and RHCE diagram representing their conformation proteins in the on red cell membrane (Daniels, 2013).

2.3.2: Weak D Variants

The phrase D alteration (variant) denotes all forms of D alleles. The weakened form of D antigen is also referred as Du or "weak D". It was described in 1946 by Stratton (Daniel,2013) when he observed that red blood cells from a donor failed to clump with 20 anti-D sera nevertheless reacted variably with 12 other anti-D reagents. He concluded that the donor could have inherited a D variant from one of his parent (father). He reported the D variant as a ''new Rh allele or alternative form of D gene' 'which he named "DU" or weakened form of D antigen. (Daniels, 2013; Wagner *et al*., 2000).

In routine D typing, this weakened D form reacts weakly with some commercially acquired D antisera's and strongly when an immediate spin or 37° incubation is carried out (Direct Coombs Test/DCT). Weakened D erythrocytes have the D epitope but in few numbers per red blood cell in relation to the normal RhD positive cells.

These alleles include weak D, D mosaic (assortment) plus incomplete (partial) D. In cases of assorted (mosaic) type, the entire parts of the antigenic structure are present except that the variants within lack some parts (Wang *et al.*, 2010a). Immunization with D positive erythrocytes to persons with D variant lacking the specific allele may result to antibody production or alloimmunisation/isoimmunisation to the missing parts. With the advance in the production of human commercial monoclonal D antibodies in early 80s' resulted to the theory of mosaic being substituted with the concept of D epitopes (Daniels, 2013). Some D variants lack some or several D epitopes and this can lead to the formation of a D like antibody. These were formerly named as incomplete D. Determination of incomplete D erythrocytes using D-similar antisera formed via human immunisation with incomplete D antigens resulted in the categorization of incomplete D surface structures into various groups (over 33 variants) as shown in tables 1 and 2.They were given roman numbers ranging from DII to DVII. Investigation with monoclonal antisera and genotyping revealed various types of D variants to include; DBT, DFR, and RHCE*ceHAR, formerly known as DHAR , DVI, DIII, DIVa, IV, V,

and VI, used in the categorization of DIII, DVII, DFR, DBT, DAU-5, and DHAR. The D variants denote all forms of D alleles to include weak forms, partial and *DEL* alleles. The incidence of D variants shows variations amongst diverse races worldwide. In whites, it is 0.2-1 % which might be higher in the African population (Daniels, 2013).

Through advancement in current technology, commercial monoclonal D antibodies are able to identify most of the erythrocytes which were previously categorised as D^u via routine typing. Comparison amongst the partial D antigens and D^u, the latter was quantified as a variant D type basing on the total number of epitopes anchored on the erythrocytes surface membranes. Finally, all D^u antigens were give the phrase weak D **tables 3 & 4** (Daniels, 2013).

Table 3: showing a few D Variants associated with producing Alloanti – D (Daniels, 2013)

DII	DFL	DWI
DIII	DFR	Weak D type 1a
DIVa	DFV	Weak D type 2a
DIVb	DHAR	Weak D type 4
DV	DHMI	Weak D type 11
DVI	DMH	Weak D type 15
DVII	DMI	Weak D type 21
DAR	DNAK	Weak D type 57
DAU	DNB	DEL-5
DBT	DOL	DEL-ex 8 del

Table 4: Shows D Variants linked with low frequency antigens (LFAs) (Daniels, 2013)

D variant	Rh LFAs
DIII	DAK (RH54)
DIVa	Go^a (RH30)
DIVb	Evans (RH37)
DV	D^w (RH23)
DVI	BARC (RH52)
DVII	Tar (RH40)
DBT	RH32
DFR	FPTT (RH50)
DHAR	RH33, FPTT

2.3.4: Molecular genetics of D variants

The Rhesus Blood Group System comprises twofold associated pair of genes RHD and *RHCE* positioned on chromosome 1p36.1. These two genes *RHD* and *RHCE* share 93.8% similarity in all their introns and encrypting exons. Despite their close relation, theses RH genes are sit in opposite orientation in the chromosome organisation 5′–*RHD*-3′-3′- –5′ RHC peptide (Daniels, 2013). Each gene contains ten exons encoding 417 nucleotide (amino acids) proteins while the N-terminal methionine is cut from developed peptide (Daniels, 2013b; Rizzo et al., 2012).There are binary types of molecular mechanism associated with the production of D variants to include; deletion of exon 9 associated with several replication structures synonyms with short tandem repeat (STR) 80 (Lopez *et al.*, 2018) . The removal of exon 9 is assumed to be the source of a frame-shift p.Gly385Valfs*80 (Lopez *et al.*, 2018). This conformation brings about the termination of codon configuration in p.Ter418 resulting to an expected additional of RhD protein to 463 amino acids. The second mechanism is related to genetic alteration resulting to weak

phenotype due to omission which spreads through exon 10. This can be associated with many RH variants alleles varying from insertions, deletions and removals (**Figure 4**) (Lopez *et al.*, 2018).

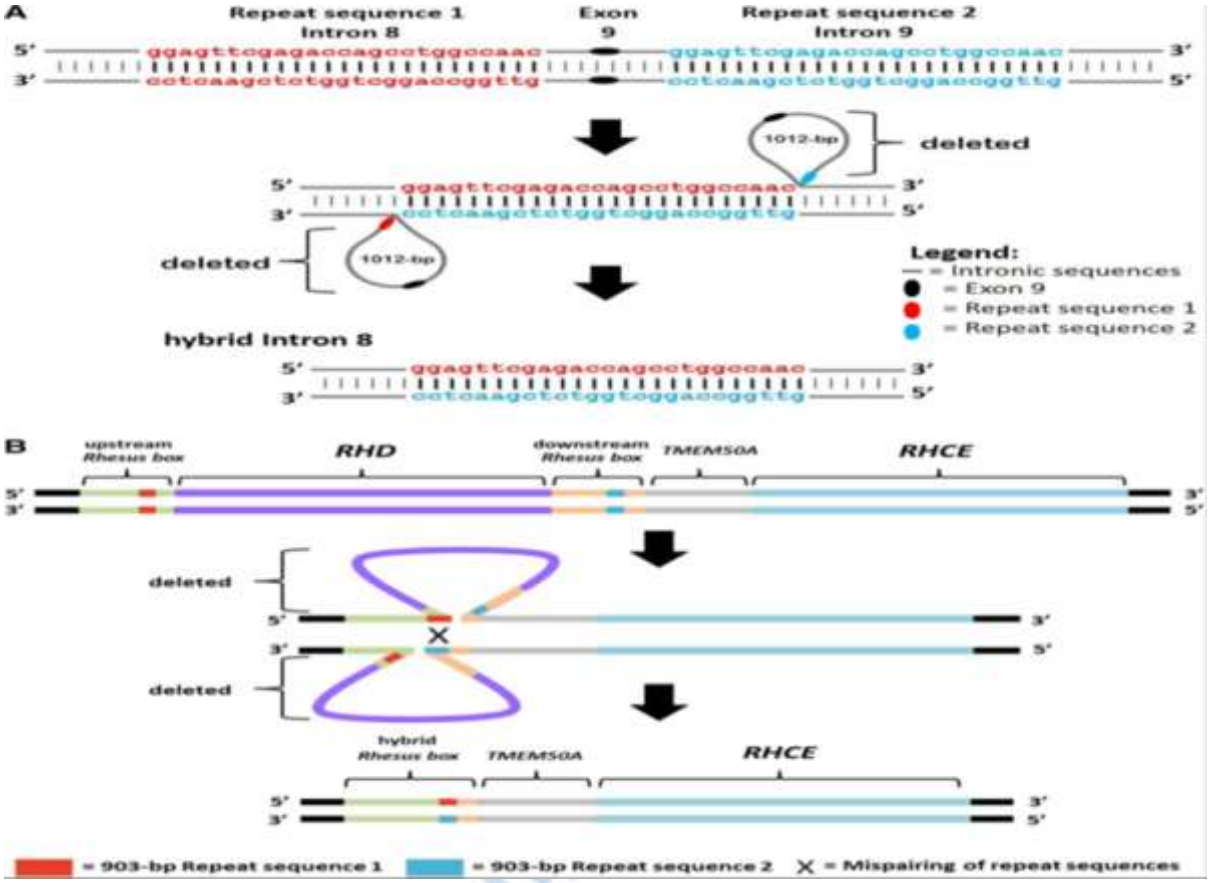

Figure 4: Proposed SSM Mechanism (A) RHD*DKG (hybrid Intron 8-a) composite of Intron 8 and 9 sequence is formed after the deletion of 1013bp, which includes Exon 9. Schematic diagram for SSM adopted from Lavinson and Gutman 23 and Fichon, *et al.* The TMEM50A gene is previously known as SMPI gene(Lopez *et al.*, 2018)

It has been shown that four kinds of RHD*DVI anchor the *RHD–CE–D* genomic combination located on *RHCE*-gene in exons 4 and 5 in type 1, 4–6 among group 2, 3–6 in group 3, and 3–5 in group 4 (Fig.4). All the four groups encrypt identical variations in the outer coils of D polypeptide each encrypting a D antigenic structure with antigenic sites specific to those of DVI. These encrypted configurations vary in the D antigen antigenic sites and manifestation of the DVI- in relation to BARC antigenic structure. Similarly, it has been revealed that there are

ten groups of *RHD*DV* that code p.Glu233Gln except DV type 5(DHK, DYO) p.Glu233Lys and *RHD*DV* type which have 4 extra alterations (changes) in exon 5. While the two categories of genetic changes defined above may explain presence of partial D variant, a single amino acid changes found inside the surface structural membrane or cytoplasm of D polypeptide are associated with the weakened forms or variants. Since introduction of *RHD* molecular typing method,145 weakened D are described with numerous sub groups (example weak D type 4·1) Rh gene database location (*RhesusBase*,2016). http://www.rhesusbase.info/,update 2018-12-02. The weak D variants can be determined via serological methods despite being linked with diverse degrees of weakness. The distributions of weak D variants show variation among different races globally. In comparison to the normal D positive red cells antigenic sites range between 13,000 - 24,000. The weak D type's antigenic sites are fewer compared to normal D positive cells (60-3800) depending on the type. Examples include DVI type 2 which have 3000 and DIII type 4 33,000 antigenic sites on the red cells respectively (Daniels, 2013).

2.3.5: Explanations of weak D and partial D

The Phrases weak D and partial D have been regularly applied to govern the management of transfused in terms of anti-D immunoglobulin administration. These phrases might be very confusing if not well described in relation to use Rhogam (Anti-D) treatment. Previously, three descriptions were used in defining the weak D and partial variants to include; Weak D antigens encompasses entirely D antigenic determinants; partial D antigenic structure lack a single or several D antigenic determinants. These terms were very confusing and difficult to link them with the serology outcome due to absence of an agglutination with some specific monoclonal antibody or method associated weak manifestation of the antigenic determinant, instead of its absence. DIIIa seems to contain totally all the antigenic determinant, but individuals with DIIIa erythrocytes frequently form D immunoglobulins/agglutinins and thus erythrocytes so their red cells must be missing a minimum of one D antigenic determinant on the surface membranes.

Persons carrying partial D antigens are capable of forming anti-D; however, individuals of weak D antigenic category do not produce antibodies. It is worth noting that absence of D immunoglobulins/agglutinins in a person is not a guarantee that another person harbouring this specific D variant, will not develop D antibodies once exposed to the D+ erythrocytes through transfusion and pregnancy (Daniels, 2013). In partial D, the RhD molecule the nucleotides alterations occur externally on the red surface sheath, while in weak D, the RhD polypeptide nucleotides alterations happen inside membrane-covering areas or the cytoplasmic helices of the polypeptide. This description was also faced the challenge of defining the exact or specific location where the amino acid residues within the RhD molecule thus redefining all these terms to one known as D variants (Daniels, 2013).

2.3.6: D Variants

The Rh D variants (weak and partial D) are very important in transfusion science. However, these terms have been applied using various definitions to include; weakened forms of D aggulutinogens, incomplete D type. Weak D contain all the antigenic parts while partial D is devoid of some D antigenic sites. DIIIa display all antigenic sites; however those persons of DIIIa on the erythrocytes surface membranes often make anti D due to lack of some D epitope (Daniels, 2013). Persons of partial D phenotype can form anti-D; while individuals of weak D type are not expected to produce D antibodies. This is the common explanation of the separation (dichotomy), though is determined by an immunological reaction. In other developments, weakened forms of D category 4•2 plus 15 more have been added in the class of weak D. Most of these weak types have been discovered and associated several patients who have already formed alloanti-D (Daniels, 2013).

In partial D, the RhD polyketide contain all the nucleotides changes on the external part of the cell membrane, however, in weak D the polyketide consist of one or more nucleotide changes inside the membrane-crossing areas or the cytoplasmic spheres of the polypeptide (which are not displayed outwardly) thus making it difficult to describe the exact site of the

nucleotide residues of the D molecule inside the membrane. This has resulted into diverse models predicting unrelated sites for various amino acids (Daniels, 2013).

The D, DAR and weak D type 4·2 is linked to persons who are alloimmunized with D antibody. They pose three identical nucleotides changes that differ with only differing only by one analogous mutation. Subsequently, the phrase DAR and weak D type 4·2 signify D allemorphs with alike phenotypes and nearly same genotypes. This conveyed the term D variant (Daniels, 2013).

2.3.7. DEL V-ariant

DEL has been described and categorised as a weak form of D. It is not detectable by the usual haemagglutination techniques. However, its presence can be determine though adsorption and elution methods. It has been observed that the antigenic site density for each erythrocyte (red cell) is approximately 36 although in many situations they are below 22 (Gassner *et al.*, 2005). Studies conducted in Northeast Asia, indicates that D– prevalence is 10% to 33% basing on the standard serological tests. The *RHD* gene is accountable for DEL variant. The DEL variant occurrence is associated with an alteration change in the 3′ molecule (aa) exon 9 (c.1227G>A, p.Lys409Lys), resulting to the removal of exon 9 from the mRNA (Daniels, 2013). The existence of DEL in Asians cannot be wholly linked with anti-D formation. The most familiar *DEL* allomorph the Caucasians encrypts p.Met295Ile (weak D type 11). The next frequent allele is linked to gene modification in the DNA sequence occurring at the border of an exon and an intron *RHD* (IVS3+1g>a) (Daniels, 2013; Gassner *et al*., 2005; Wagner *et al*., 2000).

2.3.8: Immunogenicity of D Variants

The D is very immunogenic placed only second to the ABO antigens. Approximately 20-30% of D negative (D–) persons who are infused with large quantities of D + red cells become immunised with D isoantibodies. Persons known to have several D variants are at risk of forming D alloantibodies after receiving D+ red cells. Frequencies of weak D show variation in different races. In whites, the most frequently D variant associated with anti-D production

is DVI. In black Africa race, many variants are of DIIIa and DAR types. Babies and infants born of women immunised with anti-D from D variant red blood cells have also suffered from severe HDFN. About 0.2 – 1. % of Caucasian express weak D. Persons with weakened D form Type 1, 2 and 3 are not in danger of producing D antibody. It is necessary that donors of red blood cells capable of triggering immunisation in a D- patient are marked D+. The D antigen should be determined on each blood donation (Daniels, 2013).

The Rhesus blood group D phenotype necessity determination in every donation of blood. Other D variants, including DEL, are undetectable via the agglutination methods such as coombs tests (indirect antiglobulin test/ICT). There is confirmation about DEL causing primary immunization to D- receipts via transfusion, as well as from weak forms of D, such as weak D type 2 weak D type 26 (Daniel, 2013; Gassner et al, 2005). *RHD*DEL* is naturally related to *RHCE*Ce* or *RHCE*cE* thus it is essential to transfuse D− C− E− erythrocytes to D−recipients, particularly women of child bearing age. Both serology and genotyping techniques are important to define the presence of D structure in D− donors for safer transfusion therapy. This will ensure that D− patients with DEL red cells are not given D negative young mothers and those of childbearing age (S. M. *et al.*, 2009., Daniels, 2013).

2.3.9: Rheumatic Heart Disease Linkage with RHD

Rheumatic heart disease begins as a sore throat infection caused by bacteria known as *Streptococcus pyogenes* (group A streptococcus). This bacteria is simply transferred from individual to another individual in similar ways as of other upper respiratory tract infections. These illness are commonly found with children. In most of the people experiencing this type of infection, recurrent strep illnesses result to an immunological response where the immune system ends attacking own tissues /organs body including worsening and damaging the heart valves a condition referred to rheumatic heart disease https://www.who.int/news-room/fact-sheets/detail/rheumatic-heart-disease

Facts sheet from WHO about Rheumatic heart disease to include the following

- Rheumatic heart disease is the greatest frequently acquired heart disease in persons below age 25.
- Rheumatic heart disease is responsible for above 288 348 deaths annually and most of them live in low- or middle-income countries.
- The disease consequences are due to destruction to heart valves initiated by one or several episodes of rheumatic fever, an autoimmune inflammatory reaction to throat infection with group A streptococci (streptococcal pharyngitis or strep throat) mostly in children and can lead to loss of live or life-long disability.
- Rheumatic heart disease are preventable if detected and managed early with antibiotics

A recent study carried among Black African persons has shown that there is association between RHD in the vulnerability of Rheumatic heart disease. This was linked to a new study subject susceptibility loci limited to Black African persons and an significant hereditary element to RHD vulnerability in people of African origin (Machipisa et al., 2021)

2.3.10: D Variants Genotyping

The Rhesus D antigen is located on chromosome 1, (p36.13–p34.3) with numerous alleles. The RH blood framework is translated by twofold closely related analogous genetic materials, *RHD* and *RHCE*. They are associated with five core phenotypes (D, C, E, c and e). *RHD* encrypt D antigenic motif and *RHCE* (C, E, c, and e) antigens. Numerous *RHCE* allemorphs existence is now revealed via molecular methods. RhCE variants have been associated SNP or several nucleotide substitutions within the *RHCE* gene; this has been linked to *RHCE-D-CE* hybrid alleles in the DNA location. (Bugert *et al*., 2009).

The *RHD* molecular basis is now known (S. M. *et al*., 2009., Daniels, 2013). The main genetic base for the D antigen-negative is the total removal of *RHD* gene. Currently more than 200 D variants have been identified via molecular genotyping NGS. The molecular basis of *RHCE* variants has been linked to a SNP and multiple nucleotide substitutions, single exon and multiple exon replacement (Bugert, *et al.*, 2009). Information on the molecular bases of the

RHCE forms the basis in the prediction of ce variants from genomic DNA. Determination and identification of red cell polymorphism at gene level is crucial in the transplantation and transfusion treatment, creating red cell gene repository, in matching red cells unit for recipients with a rare type and those who require massive transfusions (Flôres, *et al*., 2014)

2.3.11: Medical significance of D variants in patients

D alleles are of clinical significance both in donors and patients. They have been described to be highly immunogenic only second to ABO. One introduced to those lack them either through transfusion of D+ red cell pregnancy due to a D+ fetus and D- mother, they are capable mounting an immunological response by forming alloanti-D antibodies that result to mount a serious adverse haemolytic transfusion responses and grave HDFN. D antigen is capable of causing alloimmunisation in approximately 20–30% of D– patients transfused with many units of D+. Therefore there is need to apply further testing with a D panel to supplement the coombs (ICT) to adequately identify the D variants and also molecular genotyping application for resolving complicated cases associated with anti-D alloimmunisation (Daniels, 2013).

2.4: The rare red cell variants (C,E, c, e, MNS, Kell, Kidd and Duffy)

2.4.1: Introduction

The erythrocytes genes are highly polymorphic. (Storry *et al*., 2011). Most studies associated with red cell genes continue to reveal new allelic variants mainly due the advance in molecular genotyping technology. The word rare means less than 1% (not common). The rare red cell variants are those that lack or miss an antigen which is common in the majority of the population, or they express an antigen which most people lack. This is mostly expressed in some blood types to include; Rhesus C, E, MNS, Kell, Kidd, as well as Duffy. Serological detection of these rare variants requires specific reagents supported by molecular analysis (Anstee, 2009).

These uncommon alleles are also linked to clinical importance in pregnancy, transfusion and transplantation because they are capable of the formation of immunoglobulin/alloantibodies.

They have also been implicated in alloimmunisation include. The produced alloantibodies work best at 37◦C (IgG type) and are responsible for red cell destruction in pregnancy, transfusion and transplantation causing illness and in some cases death. The incidence and conformity of erythrocyte (RBC) isoantibodies differs in relation to the hereditary heterogeneity of the population (Pandey *et al.*, 2014).

2.4.2: RHCE VARIANTS

Rh (Rh) blood system is very diverse and of medical/scientific importance in pregnancy, transfusion and transplantation. Ranked at number two in the order of medical importance behind ABO. To date more than 56 antigens associated with the Rh. It has five main epitopes major to include; D, C, c, E, and e. It is under control of two genes RHD and RHCE polypeptides which account for their coding encryption. RhD and RHCE genes are interrelated, they are established on opposite alignment on the short arm chromosome 1(1p36.11). These genes are acquired as a pair each comprising ten exons with over 90% structure resemblance. Their neighbouring closeness chromosomal location has occasioned genetic material exchange producing lone or several amino acids alterations or crossbreed allomorphs due genome changes as well as build-up of new nucleotide modifications. It is estimated that there are over 700 RHD and 200 RHCE allomorphs. Numerous of the well-defined RH allomorphs are documented in persons of African race. Approximately 87% of those with anaemia associated with sickle cell disease and black race ancestry blood donors convey as a minimum one alternative (variant) Rh allomorph. This mixture results to medical challenges such as high incidences of Rhesus isoimmunisation. Rhesus immunoglobulin occur more frequently in transfused recipients compared with other blood systems. Most Rhesus alleles have been linked with late haemolytic transfusion reactions and HDFN (Sippert *et al*., 2021) .

Preferably, patients conveying Rh variants through an agglutinin to a common occurring antigens or with several frequent immunoglobulin require well-matched packed red blood cell (RBC) units. Nevertheless, giving well matched units is regularly a big task because RH

extended phenotyping is not usually available in most of the developing countries while genotyping that can aid in the identification of these the Rh variants is not affordable (Sippert *et al*., 2021).

2.4.2.1: Molecular Basis of RHCE

The RHD molecule transforms the RhD polypeptide, transporting the D antigen (RH1) whereas the RHCE gene codes the RhCE polypeptide, conveying the C (RH2) or c (RH4) and E (RH3) or e (RH5) antigenic structures. RhD and RhCE polypeptides together with RhAG molecule play a fundamental role to the erythrocyte sheath. Lack or non-appearance of RhAG polypeptide results in absence of RhD and RhCE polypeptides resulting to an unusual phenotype known as Rh null. Persons of Rh Null type can easily make isoantibodies/ alloantibodies once exposed to Rh antigens (Sippert et al., 2021).

There are four major types of RHCE allomorphs that encrypt the Ce, CE, ce, and cE antigenic arrangements. Any variations to the RHCE polypeptide molecule brings about changes in their antigenic manifestation and/or produce novel antigenic structures. The C and c antigenic locations are well-defined by four point mutations or changes at c.48G>C (p.Trp16Cys), c.178C>A (p.Leu60Ile), c.203G>A (p.Asn68Ser), and c.307T>C (p.Pro103Ser); and 2 identical replacement c.150C>T and c.201A>G (1, 21). Amongst documented alterations, only the p.Pro103Ser exchange, projected to occur within the subsequent extracellular sphere of the RhCE polypeptide is linked with the C/c immunological response or immunogenicity. The nucleic acids translated by exon 2 of the RHCE, allomorphs are alike to the ones encrypted on exon 2 of the RHD gene. Next-generation sequencing records indicate a crossbreed allomorph RHCE*CE-D (2)-CE that is closely associated with the C+ phenotype countenance. The genomic foundation for E and e eccentricities are well defined through the nucleic acid modification c.676G>C in exon 5, initiating a nucleotide replacement, p.Ala226Pro, situated in the fourth outer sphere of the RhCE polypeptide (Sippert *et al.*, 2021).

2.4.3: MNS Blood group System Variants

Landsteiner described the MNS in 1927. Currently, there are50 MNS antigens (Lopez et al 2021). It is composed of two glycophorin GPA and GPB located on the red cell membrane with four polymorphic antigens; M,N,S and s (Faria et al., 2012). GYPA, GYPB and GYPE genes display a high degree of similarity above 95%. GPA and GPB are encrypted by a pair of closely related genes *GYPA* and *GYPB* situated on chromosome 4 (4p28-q31) (Faria, *et al.*, 2012). This system is polymorphic with a point mutation (SNP) that regulates S/s and M/N allele variations. Persons devoid of the *GYPB* genes lack S, s and U agglutinogens. Nevertheless, S–s–U+var type displays U antigenic and lacks S and s antigenic structures on the erythrocytes external sheath. It is linked to changes in exon 5 [*GYPB*NY* alteration] on the other hand intron 5 [*GYPB*P2* variant]. The alterations are mostly common in African race and are linked to a partial or full deletion of exon 5 of *GYPB* gene (Faria, *et al.*, 2012). This system is associated with adverse immunological responses and perinatal HDFN. Commercial U immunoglobulin's (anti-sera) availability is a challenge making it difficult to perform a routine compatibility test thus use of genotyping can be applied to reveal the variants (Faria, *et al.*, 2012).

2.4.3.1: NEW MNS antigens acknowledged by ISBT

International Society for Blood Transfusion (ISBT) has recently recognized and documented four new MNS antigens bring the total to above 50. These antigen qualified and met the ISBT antigens recognition criteria. They include; SARA (MNS47), KIPP (MNS48), JENU (MNS49) and SUMI (MNS50)

2.4.3.1.1: SARA (MNS47)

A recent study among routine donor's red cells that were used routinely as the panel for antibody determination showed agglutination with serum sample from a recipient. Serology results had indicated that the antigen was newly acquired. This antigen was initially called "SARAH" but is now known as SARA. On applying complete genomic sequencing on SARA+

individuals, it was revealed that that *GYPA* c.240C>T was the genetic foundation for the SARA antigen. In 2015, the ISBT Working Party on Red Cell Immunogenetics and Blood Group Terminology (RCIBGT) allocated SARA as MNS47. As of recent studies two case of HDFN have been reported to be associated with SARA antigen .(Lopez et al., 2021).

2.4.3.1.2: KIPP (MNS48)

Some red blood cells coming from a German donor presented unusual reaction patterns with immunoglobulin of known specificity to low- occurring MNS antigens. This phenotype was named GP.Kip. A laboratory information specified that 'Kip' is the abbreviation for the name Kippenhah and was also named as in an Australian blood service. DNA sequencing carried out in Australian had shown that GYP***Kip* was a hybrid *GYP (B-A-B)* gene. GP.Kip is Mi^a+, conveys p.Ser51, and was distinctive from other Mia+ GYP (B-A-B) hybrid glycophorinsThe KIPP antigen on GP.Kip is acknowledged by two anti-Hop (+ Nob) antisera, Anek and Raddon. Another Mi^a+ hybrid glycophorin called GP.MOT is also associated to KIPP antigen. This antigen is more common in the people of Japan (Lopez et al., 2021).

2.4.3.1.3: JENU (MNS49)

A thalassemia patient from Thai was infused with Red Blood Cells. Post transfusion evaluation revealed that the recipient had developed several alloantibodies to include; antibody (ies) E, c, Jk^b, anti-S and a common occurring type GPB. Epitope profiling investigation applying twelve-mer polypeptide s, signifying the extracellular area of GPB, revealed that an alloantibody present in the recipient plasma Phenotyping and genotyping indicated the recipient was GP.Mur homozygote (GP.Mur/GP.Mur). GP.Mur/GP.Mur persons do not display usual GPB and, consequently, JENU-negative. These individuals can yield alloantibodies to GPB as well as anti-JENU (Lopez et al., 2021).

2.4.3.1.4: SUMI (MNS50)

A recipient serum reacted to red blood from a blood donor through usual routine cross matching test but failed to show any agglutination when subjected to antibody identification red blood

cell panel. Consequent serology investigations were carried out and named the present antigen-SUMI. An antibody SUMI monoclonal type forming cell line was developed. Anti-SUMI was applied in the screening of 541,522 donations and recognized 23 were SUMI-positive. Molecular genotyping results indicated that all the 23 individuals conveyed a single polymorphism at *GYPA* c.91A>C (p.Thr31Pro). SUMI has been established to be is a low-occurring antigen on GPA with a prevalence of 0.0042% among the Japanese donor population. SUMI antigen was assigned number MNS50 by the ISBT Working P arty on RCIBGT.(Lopez et al., 2021)

2.4.4: Kell System Variants

The Kell blood group system is given the ISBT number 006. It has been grouped amongst the most important blood groups in transfusion, pregnancy and solid organ transfer medical care. Kell was recognised in 1946 and Levine and colleagues later revealed its antigen and associated alleles/variants in 1449. It is highly immunogenic and is linked to adverse effects in transfusion and pregnancy. Kell system alloimmunisation has been incriminated in influencing against HLA antigens in solid organ transplants (Holt *et al*., 2020).

The Kell System comprise 36 antigens. Most of the Kell antigens are expressed antithetically showing variations among the phenotypes of high and low prevalence such as KEL2 (k)/KEL1 (K), KEL4 (Kpb)/KEL3 (Kpa)/KEL21 (Kpc), KEL7 (Jsb)/KEL6 (Jsa), KEL11/KEL17, and KEL14/KEL24. The others have no known antithetical partner and are called para-Kell. Among the various Kell antigens, K/k (KEL1/KEL2), Kpa/Kpb (KEL3/KEL4), and Jsa/Jsb (KEL6/KEL7) which have been listed to be medical importance the most important (Mattaloni *et al.,* 2017; Yu *et al.,* 2001).

These antigens are among others that are associated with neonatal anaemia and transfusion reactions. They are carried on a single pass glycoprotein molecule comprising of 19 exons, crossing greater than 21 kilo bp genetic Material weighing 93 kDa in the *KEL* gene. The Kell phenotypes are products of a single alterations (Anstee, 2009; Yu *et al*., 2001). The

transmembrane area of Kell glycoprotein is under the control of the *KEL* gene exon 3. A replica devoid of exon 3 is predicted to carry unexpected termination code (stop codon) which is responsible for removing the transformation of C-terminal section resulting to Kell-null phenotype or Ko erythrocytes (Yu, *et al*., 2001). Around eleven Kell antigenic allele have been revealed. A number of Kell antigens demonstrate racial linkages. Js^a, is a rare occurring allele with a highest frequency of almost 20% in Blacks (Yu, *et al.*, 2001).

The molecular bases of almost all Kell phenotypes have been revealed. The *KEL* cDNA polypeptide is composed 732-nucleotides. The transformation site for the *KEL* gene located on chromosome 7q33 covering more than 20 kilobase of exon 19. Kell Gene is associate with its antigens and various phenotypes to include; McLeod, Gerbich-negative, Kp^a, and Kmod. It is necessary to perform extended serological phenotyping in donated blood to identity these antigens in order to grow unusual blood donor inventory in the occasion a necessity might arise warranting an antigen-negative red cell units. Also use of molecular genotyping will aid to reveal their frequency and offering antigen negative units when required (Yu *et al.*, 2001).

2.4.4.1: Kell null ((K0) Phenotype Medical Significance

To date, approximately 35 Kell antigens have been documented in the ISBT website. Of the 35, K/k (KEL1/KEL2), Kpa/Kpb (KEL3/KEL4), and Jsa/Jsb (KEL6/KEL7) are regarded as the most important in regard to their immunogenic character. Little has been documented on the null (K0) type. KEL null phenotype is a person who lacks any expression of the Kell antigens. However, these persons are capable of forming alloantibodies against many antigenic determinates in the Kell molecule if exposed via blood infusions, pregnancy and solid organ transplantation. Owing to the fact that the KEL null phenotype is a rare type not much has been documented about the antibody against the KEL null type medical importance especially in relation to haemolytic disease of the foetus and new-born after blood transfusion or during or after gestation in women of child bearing age. The KEL null has been implicated in causing high levels of bilirubin in neonates owing to the activity of the immune system causing red

blood cell damage by the formed an isoantibodies that recognise the Kell epitopes as foreign. The identification of this anti-Kell is usually accomplished by use of serology phenotyping and molecular genotyping techniques. In most cases this KEL null immunization is associated with a single polymorphism or mutation (KEL*02N.06 allele). Alloimmunisation with K null immunoglobulin is mostly linked to high levels of bilirubin, haemolytic disease of the foetus, and new-born thrombocytopenia with serious pre-natal low haemoglobin levels. This phenotype despite being of low prevalence should be determine especially in antenatal cases and to ensure measures are taken to reduce pre-natal risks (Mattaloni et al., 2017).

2.4.5: Kidd System Variants

The Kidd system was revealed in 1951. JK is a glycoprotein composed of two main antigenic structures, Jk^a and Jk^b and a low frequency variant Jk3 which is a null type and very rare. There are various phenotypes associated with this system to including; Jk (a+b-), Jk (a-b+), Jk (a+b+), and Jk (a-b-) The Kidd protein is a 43-kDa, with 389-nucleotide molecule spanning the erythrocytes membrane area 10 times. It is also associated with the transportation of urea both endothelial cells and red cells. In order of clinical significance, it has been involved in immunological aspects of HDFN and transfusion reactions (Liu, *et al*., 2012; Anstee, 2009; Lawicki, *et al.*, 2017). The inheritance of Jk^a and Jk^b is co-dominant. The unusual null type Jk(a-b-) is due to an inactive concealed variant (Liu, *et al*., 2012).

The gene responsible for encoding the Kidd glycoprotein is known as *HUT11/UT-B/JK* (*SLC14A1*) positioned on chromosome 18q12-q21 (Lawicki *et al*., 2017). Cloning of the Kidd gene has enabled the identification of Kidd variants/alleles via molecular genotyping (Anstee, 2009).

The JK antigenic determinates act as carriers or are involved in transportation and are distributed on the red cell surface sheath and endothelium of the Kidney. Nevertheless, it has been noted that they are also found in an extensive range of solid tissues such as renal medulla, vasa recta, and the kidney tubules epithelial cells where they play a key function of maintaining

osmotic homeostasis between the erythrocytes and renal medulla by controlling the inflow/outflow of urea within the erythrocytes and tubular lumen (Sanford et al., 2015).

The Kidd (JK) system two major phenotypes to including Jk^a and Jk^b antigens have been implicated in formation of antibodies once introduced to those lack them. The first antibody to be described was Jk^a in 1951 by Fred H. Allen, Louis K. Diamond, and Beverly Niedziela, in a serological mismatch between delivery where the mother was Jka- and the new-born was and Jk^a+ that caused a serious condition hemolytic disease of the fetus and newborn (HDFN). The serum of the mum was found to contain immunoglobulins directed against the neonate erythrocytes. The immunoglobulin recognized a specific immunoglobulin or alloantibody which was later associated with Jka. Later in the year 1953, antithetical alloantibody was recognized Plaut et al and named as Jkb. In 1959, the JK null phenotype was documented following a mild hemolytic transfusion adverse event in which the recipient displayed immunoglobulins to Jka and Jkb which came to be known as antibody -Jk3 (Sanford et al., 2015).

2.4.5.1: JK Immunoglobulins Characteristics

The most documented antibodies of this system include, Jk^a and Jk^b types. They are classified as IgG type especially in the sub-classes of IgG1 and IgG3. The IgG antibodies have the ability to bind complement C3d, consequentially causing intra- and extravascular haemolyses that is specific to serious plus post haemolytic transfusion reactions, haemolytic disease of the foetus and new born including several kinds of transplantation rejection in recipients of solid-tissues transplants. Jk immunoglobulins presence is associated with risks of post transfusion haemolysis due to their capacity to agglutinate JK antigen reactive red blood cells as well their ability to fix compliment which ends in causing associated widespread intra- and extravascular haemolyses. Post red cells transfusion adverse events resulting to anti-Jka antibodies are linked to isoimmunization emanating from earlier transfusion of differences in blood and blood

products. However, it can also result in cases of nonexistence related transfusion account owing existing natural arising anti-Jka antibodies (Sanford et al., 2015).

2.4.5.2: JK Antigens and immunoglobulins Determination techniques

Serological methods using commercially acquired antisera's are in use in the determination of the presence of Jk^a and Jk^b phenotypes on the donors and recipients red cell membranes. Phenotyping donated red cells using coombs test (indirect antiglobulin test) is beneficial in the selection of antigen free pints for infusion to recipients who are already alloimmunized with Jka or Jk^b antibodies through transfusion, pregnancy plus solid tissues transplants. Extended antigen profiling is as well carried out to check the existence of isoantibodies in recipients or donated samples. This is accomplished by carrying out a by matching donated erythrocytes with recipients serum applying an indirect antiglobulin test. If clumping occurs, the results are interpreted as reactive test showing the existence of a significant antigen. In case of absence of an antigen, there will be nothing for the antibody to attach to and thus no clumping of erythrocyte thus a negative reaction meaning that there is no offending antigen(Sanford et al., 2015).

In some circumstances, recognition of existing JK immunoglobulins may prove a bit difficult especially their presence drops lower than the expected titration levels of detection in the recipient sample (plasma) as frequently observed. Nevertheless, their low detection levels does not mean they are limited in their immunological provocation which result to adverse transfusion are capable reactions. Since they are Ig Immunoglobulins they react best at warm temperatures in combination with coombs reagent. For their accurate detection, their determination requires to be tested at 37 ^{o}c using the antihuman globulin (AHG). Once reacted in the warm temptation and use of AHG, these antibodies will be able to attach to specific antigens on the red cell surfaces and an agglutination will be visualized. At times use of enzymes to treat the erythrocytes with enzyme to allow digestion of the sialic acid on the red cell surface membrane to expose the existing antigens for the uptake by the immunoglobulins.

Augmentation substances such as AHG, low ionic strength saline, Enzymes are pivotal in the determination of JK immunoglobulins in the reduction of adverse post transfusion immunological or severe haemolytic transfusion reactions (Sanford et al., 2015).

In disease association, the JK immunoglobulins have been implicated to renal transplant failures. (Sanford et al., 2015). Studies on JK phenotypes and associated antibodies have shown that they act as insignificant human leukocyte antigens (HLAs) after kidney transplants since they are also present on the renal endothelium. Consequently it is crucial to identify them in kidney recipient's pre and post transplantation (Sanford et al., 2015). .

2.4.6: Duffy Blood System Variants

Duffy blood system discovery was recognized as Cutbush in 1950. The Duffy antigens (Fy^a, Fy^b) are glycoproteins that spans the red cell membrane 7 times (Höher *et al*., 2018). They show variations in different populations. The *FY*B* allele is common in Africans (0.981), but not in East Asians (0.077). However, *FY*A* allomorph is widespread in East Asians (0.923), but rare in Africans (0.019) (Höher, *et al.*, 2018).

The FY antigenic structures are transported on the FY glycoprotein coded by the Duffy gene. FY glycoprotein similarly identified as Duffy Antigen Receptor for chemokines (DARC) (Höher, *et al*., 2018) The major common roles played by DARC include; maintaining homeostatic levels of circulating chemokines, regulating chemokine increase within tissues and the blood in order to resolve entry of phagocytic cells from blood vessels into tissues during an immunological reactions. Research has shown that there is some linkage into its part in inflammation-linked pathogenesis and malignancy (Anstee, 2009; Höher, *et al*., 2018).

It also plays a major role as a portal of entry for malarial parasites into human red blood cells. While Plasmodium falciparum uses a series of receptors on the surface of human erythrocytes to invade them, *Plasmodium vivax* and *Plasmodium knowlesi* depend on an interaction with the Duffy antigen (Anstee, 2009; Höher, *et al*., 2018).

The Duffy system genome is situated on chromosome 1 q21-q25. It is encoded by *ACKR1* gene (*DARC* or *FY*). *ACKRI* gene is associated with two main variants *FY*A* and *FY*B* alleles conveyed codominantly over null variants $FY*B^{ES}$ and $FY*A^{ES}$. These combination are responsible for the production of various phenotypes to include; Fy (a+b+), Fy (a+b−), Fy(a−b+) and Fy (a−b−) (Höher, *et al.*, 2018).

Duffy structure encodes a seven membrane spanning peptide with a mutation associated with one nucleotide alteration at location p.42 in the outer layer of amino-end region (Anstee, 2009). Duffy null trait (FY (a−b−) is common in Europe, Asian and among blacks. The FY (a−b−) type have been described in Australian specific population. These rare phenotype has several molecular backgrounds and can be revealed by molecular genotyping (Anstee, 2009). A single nucleotide variant c.265C>T in the *FY* is responsible for the Duffy weak appearance of Fy^a and Fy^b antigens(Höher, *et al.*, 2018).

2.4.7: Dantu Variants

Dantu (MNS25) is a rare aggulutinogens of the MNS system. It is carried as a hybrid glycoprotein on the erythrocytes membranes and is genetically inherited (B. O. *al et*, 1987). Dantu antigen is linked to the weak (MNS4) antigen. The frequency varies in different populations. It has been shown to be 0.05 among the Black Americans and less than 0.01 in the German population. The main composition of M-or N- active sialoglycoprotein (glycophorin A, GPA) in Dantu/U heterozygotes has been demonstrated to be reduced by around 57% and S-or s-active sialoglycoprotein (GPB) by roughly 51% (Blumenfeld *et al.*, 1987; Dahr *et al.*, 1987; Huang & Blumenfeld, 1988). Dantu-positive cells show a protein enzyme -safe GP (B-A) weighing 29 KDa. In this study the aim is to establish their presence and frequency via molecular genotyping.(Blumenfeld, *et al.*, 1987; Dahr *et al*., 1987; Huang & Blumenfeld, 1988).

CHAPTER THREE

RESEARCH METHODOLOGY

3.1: Introduction

This section details the thesis design, area, population (donated donor samples) sample size determination, sampling procedure, inclusion plus exclusion criteria, reagents preparations, laboratory method and ethical consideration.

3.2: Study Design

A cross sectional experimental research strategy was employed to accomplish the study. Limited blood group serology for ABOD and molecular genotyping was employed to determine the red cell variants in selected donated whole blood samples from voluntary African donor population in Kenya.

3.3: Study Area & Sample analysis centers

This research was accomplished at Blood Grouping Laboratory- KNBTS (-1.29965, 36.8118), Map Link to Kenya National Blood Transfusion Services/Tissue and Transplant Authority site where limited automated serological determination of ABOD red cell antigens was carried and samples for DNA extraction identified. Genomic DNA manual extraction was conducted at National Malaria Reference Molecular laboratory at National public Health Laboratories-Kenya (-1.30412, 36.80707) Link to National Public Health Laboratories Services, and sequencing analysis at Red Cross blood service laboratory, Queensland, Australia (-27.45007, 153.01267) Link to Australia Red Cross Lifeblood Brisbane (appendix I)

3.4: Study Population

The research populace were blood donors' donated blood samples for routine blood grouping procedure.

3.5: Sample Size Determination

The study employed Slovin's Formula Sampling Techniques-1960 – (Updated December 14, 2020 By Stephanie Ellen) where the sample size was 92 samples

$$n = N/ (1 + Ne^{2})$$

Where:

- n = Number of samples=120
- N = Total population = 0.5%
- e = Error tolerance (level)

n = 120(total samples)

N = 5% (0.5) (CI)

$120/ (1+120*0.5^2) = 120/ (1+120*0.5*0.5) = 120/ (1+120*0.025) = 120/ (1.3075)$ 1.3075=91.78

Sample size =92

3.6: Sampling Procedure

Purposeful sampling was employed during this study. All samples collected in ETDA tube, some samples (70) were RhD positive while the rest were weak D positive, D negative, non-reactive for transfusion transmission infections (TTIs), with no haemolysis during the automated serological determination were selected for this study by applying Slovin's Sampling Techniques.

3.7: Inclusion and exclusion criteria

All blood samples collected in EDTA tubes that met the sampling DNA extraction requirements and were available at the KNBTS blood grouping laboratory record were selected for this study. All blood samples in EDTA tubes that failed the required inclusion criteria during DNA manual extraction.

3.8: Experimental and laboratory techniques

3.8.1: Reagents Preparations

All reagents preparation was performed following the manufacturer's instructions. Automated serology blood typing reagents were commercially acquired and thus no preparation was required during antigens identification procedure. Manual DNA extraction reagents required

reconstitution and dilution; lyophilized QAIGEN Protease was reconstituted with 5.5mls and protease solvent, BufferAW1 (98mls) and Buffer AW2 (66mls) concentrates were diluted with 130 mls and 160 mls of absolute (100%) ethanol respectively. Buffer AL (lysis buffer) did not require any dilution. DNA quantification, Illumina DNA prep with Enrichment and DNA sequencing (*QIAamp DNA Blood Mini Kit (250) 51106 from QIAGEN - Sample to Insight | Biocompare.Com*,2016).

3.8.2: Automated Blood Group (Serology)

During the performance of this procedure, all steps and processes involved were treated as infectious and thus the quality and safety protocols were observed all the way from start to completion. Automated serology blood grouping is a procedure of determining the presence of blood group antigens using an automated platform and commercially acquired antisera. The principle is based on haemagglutination/solid phase technology. Solid phase skill is built on the principle of solid-phase red cell adherence (SPRCA) used to identify antibodies by employing either an indirect or direct antiglobulin procedure. In indirect antiglobulin procedures, known antigens are bound to the surface of polystyrene microtiter plate wells which capture specific antibodies from the samples being tested. Subsequently a brief washing step, an anti-immunoglobulin coated Indicator Red Cell is applied to determine the existence of the present immunoglobulin/agglutinin.

This study employed automated blood grouping to establish the presence of ABO, RhD and weak D antigens from donor blood samples that had already been used for routine work. The procedure was carried out using model Galileo Neo Gammar platform (Serial No; 5030090749 Germany) and commercially acquired immucor blood grouping reagents to aid the identification of the ABOD and weak D antigens. Prior to running the procedure, all materials and reagents and equipment to include; bar coded round-bottomed microplates, labels, Barcode scanner, Barcode printer, Neo sample carrier, Sample diluent, Diluent container, System concentrate, Immunoclone Anti A IgM, Anti B, IgM, Anti AB IgM 10. Anti-D Novaclone,

Immunoclone Anti D Rapid, Immunoclone Rh Hr control, Capture Indicator Red cells, Reference cells kit- A (1), B, Capture Liss, corQC Extended Standard cells, Capture-R-Select plates, Gloves and samples were retrieved from specific storage such as the cold storage (4^0c), and placed at 25^{o}c. For safety measures protective personal equipment were worn appropriately. The System liquid concentrate was prepared as per the reagent insert (dilution of immucor 500ml system liquid concentrate with de-ionized water in a ratio of 1: 9) ready for use as wash solution. The samples were placed in the sample racks, controls in the specific racks and reagents respectively. The Neo equipment was switched on and primed using the system liquid to give it an initial wash run to ensure the probes were not clogged and were delivering the right volumes in preparation for the quality controls and samples run. Once the Neo equipment was primed, the program quality control (QC) was selected in the computer monitor. The QC materials were loaded as per instruction displayed on the monitor. Having loaded all the QCs materials and program was switched on and left to run for fifteen minutes, The QC the results were displayed on the computer screen and grading visualised via an inbuilt camera. Once all the QC materials passed the run, the samples were checked for integrity, volume (4mls), centrifuged for 1 minute at 1000 rounds per minute (rpm), and manually loaded on the samples carriers lanes on of the machine. The ABOD commercially acquired reagents were on the resources program, and the start program for sample run initiated. The first results were displayed after 12 minutes followed by the subsequent ones within I minute interval. The results were displayed as positive or negative and a grading program was also on board (inbuilt in the platform) to assist in grading the produced results. The results were reviewed and interpreted. Presence of agglutination indicated that the test was positive one or there was presence of the antigens for the corresponding antibody. The absence of agglutination indicated a negative or absence of the antigen for the corresponding antibody. For all the samples that were identified as weak D, the capture assay program was selected from the machine programs and initiated. An equal number of capture plate strips were prepared by removing the required

number of strips from the plate foil and fixed on the strip plate holder. The strip plate holder was loaded on the sample lane on the neo platform. The capture assay reagents were loaded as per the platform instructions and the program initiated. After twelve minutes, the results were displayed, graded and reviewed for final documentation. Agglutination meant that there was presence of the antigens for the corresponding antibody; and absence of agglutination indicated a negative test or absence of the antigen corresponding to the specific antibody as indicated in **table 5** and Appendix II (*Immucor_NEO-Brochure_US_web.Pdf*, 2015).

Table 5: Showing Agglutination Grading (SOP on Grading of automated serological blood typing)

Grading of Agglutination reactions		
Grade	Significance	Interpretation
4+	1 large clog	
3+	2 or 3 clogs	
2+	Several minor clogs with clear supernatant	
1+	Several minor clogs with unclear supernatant	
w	Coarse suspension	Positive
H	Incomplete/complete hemolysis(Positive reaction)	
Zero or -	Smooth suspension (Negative reaction)	Negative

Legend frequency of agglutination reactions grading inferred from the Neo Immucor procedure to guide in the grading of results and their significance as generated from the automated serological typing.

3.8.3: Molecular Genotyping

Prior to starting genotyping procedure, there were critical steps carried out to ensure all was in place. These steps included extraction and purification of nucleic material (DNA) to purify the genomic as described in section 3.9. The method employed in the characterization of red cell

variants was the Illumina MiSeq Next Generation Sequencing technique. It combined Illumina MiSeq and v2 chemistry platforms for clonal magnification, genotyping, and statistics analysis (base calling, alignment, variant calling, and reporting) at the same time. Variant Call Files (VCF) and binary Alignment Maps (BAM) for each sample were generated using MiSeq reporter software as per the blood group target manifesto (Bugert, *et al*., 2008; *MiSeq Reporter Training*, 2020.; Voelkerding, *et al*., 2010)

3.8.4: DNA Extraction and Purification Spin Procedure

Prior to starting the procedure, all samples and reagents were from the cold storage (4^{o}c) and placed at room temperature. All equipment (micro centrifuge and water bath) were switched on and set at 25^{o}c and 60^{o}c respectively. Genomic DNA manual extraction was done using QIAamp® Blood Mini Kit (51106); extraction and purification was achieved via Micro-centrifugation to recover DNA materials from whole blood samples(QIAamp DNA Blood Mini Kit (250) 51106 from QIAGEN - Sample to Insight | Biocompare.Com, 2016; www.qiagen.com/HB-0329, 2018; *Schoeman et al*., 2017, 2018). All the plastic micro centrifuge tubes 1.5 (V-shape) and 2 (U-shaped) millilitres tubes were appropriately labelled with unique numbers linking them to the designated donor samples numbers. 20µl of QIAGEN Protease (Lot No 16003717-Germany) was pipetted into all the labelled 1.5ml micro-centrifuge tubes, 200 µl of whole blood added to the appropriately labelled QIAGEN Protease micro tube, 200 µl lysis buffer AL (Lot no. 160031410-Germany) added, tubes contents mixed by votexing (using votexer model Retsch. Serial No 02-1911-Germany) for 15 seconds, placed at 56^{o}c water bath (model WB4, serial no 509022281-Ratek instrument PTY LTD) and incubated for 10 minutes, All tubes were transferred to a micro-centrifuge (Biofuge Fresco-Heraues) and span lightly for 15 seconds at 8000rpm; two hundred (200) µl of 100% ethanol added into all tubes contents to allow DNA precipitation, vortexed for 15 seconds and span briefly (15 seconds) at 8000rpm. The tube contents (mixture) was applied using a micropipette (1000µl) to appropriately labelled QIAamp mini spin column (in 2ml collection tubes), capped and

centrifuge at 8000 rpm for 1 minute. The QIAamp mini spin column with contents were manually transferred into new appropriately labelled 2ml collection tubes; 500µl buffer AW1 (lot no. 160034099-Germany) was added using a micropipette (to keep pH stable), capped, sealed and centrifuged at 8000 rpm(to remove drops from the lid) for 1 minute. The mini columns were placed into clean labelled 2 ml tubes, 500µl buffer AW2 (lot no. 160034497-Germany) added, capped, sealed and centrifuged at 14,000 rpm for 3 minutes. The mini spin columns were placed into new appropriately labelled 2 ml collection tubes, spun at 14,000 rpm for 1 minute. This was then placed into new 1.5ml appropriate labelled tubes. 200 µl Buffer AE (lot no. 160028092-Germany) added and incubated at 25ºc for 5 minutes to elute DNA; tube contents (genomic DNA) were centrifuged at 8000 rpm for 1 minute, manually arranged in labelled cryoboxes and stored at -70oc freezer and shipped to Australia for DNA sequencing as described in section 3.9 (Schoeman, *et al.*, 2018).

3.8.5: DNA Material Shipment

The shipment of biological materials prepared in section 3.9.4 above outside the country (Kenya) required authorization and an export permit from the ministry of health. An export permit to ship gDNA materials to Australia was requested from the ministry of health and Pharmacy Poisons Board (PPB). These were issued (Appendix III). An excel sheet for 120 gDNA materials was prepared and emailed prior to exportation. Once the shipment was received in Australia laboratory, they were entered into the Kenya data set register and gDNA quantification carried out as described in section 3.9.5.2 to establish the quality and amount in preparation for subsequent procedures. This was used to determine if the required volume of DNA was available.

3.8.5.1: Genomic DNA (gDNA) Quantification and Quality Control (QC)

Genomic DNA Quantification procedure was done using Qubit BR assay adhering to the user guide instructions manual (*MAN0010876_QRC10876_Qubit3.0_QR_RevA_08Oct2014.Pdf*;2014) The method involved

setting up two assay tubes (standard and sample); preparation of Qubit® Working Solution (Qubit® reagent 1:200 in Qubit® buffer) and 200 µl of Working Solution for each standard and sample. Thin PCR tubes were labelled for standards (3 tubes for protein assay) and one for each sample. All assay reagents and samples were placed at room temperature prior to starting the procedure. 190 µl working solution was pipetted into to the standard tubes, 10 µL of standard was added. 190 µl working solution was also pipetted into the sample tubes followed with 10 µl of the appropriate sample. The total volume of all tubes was 200 µl. All the tubes contents were vortexed for 3 seconds, incubated at room temperature for 2 minutes (15 minutes for the Qubit® protein assay); placed in the Qubit Fluorimeter and readings recorded as per Qubit® manual. Of the 120 gDNA samples, 108 satisfied the quantification criteria and were marked for the DNA library generation while 12 failed the quantity requirement (< 2ng/ µl = not suitable) thus, were not sequenced (MiSeq Reporter Enrichment Workflow Guide, 2020; MiSeq Reporter Generate FASTQ Workflow Guide, 2020); *MAN0010876_QRC10876_Qubit3.0_QR_RevA_08Oct2014.Pdf*;2014).

3.9.5.3: DNA Library Preparation (Illumina DNA prep Enrichment)

The DNA library was generated from the gDNA (108) that satisfied the quantification criteria and quality checks. The process of generating DNA library was completed by use of the Illumina DNA prep Enrichment (DEP) with customised probes targeting blood group genes and regulatory regions covering 38 blood group systems. The procedure was applicable for high quality gDNA inputs of 10-1000 nano gram (ng). The procedure involved generation of unique dual (UD) indexed paired DNA using Illumina DNA Prep with Enrichment (DPE); protocol requirements included library prep and enrichment reagents, probe panel, and index adapters. (*Illumina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf*, 2020; Roulis, *et al.*, 2020; Nielsen, *et al.*, 2011).

The Illumina DNA Prep with Enrichment workflow process employed a bead-based transposome combination to slice and tag gDNA. The sliced (fragmented) gDNA was labelled

(tagged) with adapter sequences, flooding (saturating) with input DNA enabling the bead-based transposome compound to break (split) a set number of DNA molecules. The DNA splitting allowed flexibility to apply a comprehensive range of DNA input to make standardized files of balanced portions. Subsequently, a restricted-cycle PCR augmented (amplified) adapter sequences to the ends of a DNA portion to enable integration of all Illumina sequencing platforms. Subsequently, target enrichment workflow was initiated and performed with pooled libraries (up to 12-plex). The pooled double stranded DNA libraries were denatured and biotinylated oligonucleotide probes were hybridized to the generated fragments. The Streptavidin Magnetic Beads (SMB) captured the specific library fragments inside the regions of concern (interest). The captured and indexed libraries were eluted from beads and amplified prior to sequencing (figure 5 and 6 Illumina workflows summaries). Figure 5 outlines the pre-PCR and PCR both manual and semi-automated steps with safe breaks where necessary while figure 6 details the automated process.

The gDNA prep with Enrichment workflow summary guides on all the phases to be followed prior to sequencing and analysis of generated libraries. The allocated time was based on 12 sample at 12-ples enrichment using an array of specific consumables for every phase (Enrichment Bead-Linked Transposomes (eBLT), Tagment Buffer 1 (TB1), nuclease-free water, 96- well PCR plate, microseal ‘B’ adhesive, micro centrifuge tubes (1.7 ml), 200 µl multichannel pipettes ad pipette tips, 20 µl multichannel pipette and tips, 96-well PCR plate, Microseal “B’, Tagment Buffer (ST2), Tagment Wash Buffer (TWB), Magnetic plate stand, Magnetic Stand-96, thermal cycler, micro centrifuge tubes, tips, Enhanced PCR Mix (EPM), Index adapter plate, Eppendorf Lo Bind PCR Plate, Agencourt AMPure XP beads (AMPure XP Beads), 5 ml, Resuspension Buffer (RSB), Freshly prepared 80% ethanol (EtOH-fresh), 96-well 0.8 ml Polypropylene Deepwell Storage Plate (midi plate) (2), Microseal 'F' foil seal, plate shaker), Streptavidin Magnetic Beads (SMB), Enhanced Enrichment Wash (EEW) (amber cap), Enrichment Elution Buffer 1 (EE1), 2N NaOH (HP3), Elute Target Buffer 2

(ET2), Enhanced PCR Mix (EPM), PCR Primer Cocktail (PPC), Illumina Exome Panel (CEX) (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis, *et al*., 2020).

In the Tagment Genomic DNA phase, Enrichment Bead-Linked Transposome (eBLT) were used to cleave (fragment) and label (tag) the DNA with adapter sequences. Prior to starting this step, 96 PCR plate was labelled, reagents prepared as per the reference guide and Tag program was saved on the thermal cycler. The reaction volumes were set at 50 µl. 2 µl DNA was added to each well, eBLT was vortexed strongly for 10 seconds; tagmentation master mix (ratio: eBLT µl 11.5 and TB1 11.5 µl) multiplied with the number of samples (108) was prepared and mixed carefully using the votexer to suspend it; plate sealed with Microseal "B' and placed on the thermal cycler and TAG program switched on; plate held in same position till the temperature reached 10°C before removal. This phase opened next step of Post tagmentation clean up (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis, *et al*., 2020; Nielsen, *et al.*, 2011; Schoeman, *et al.*, 2018; Schoeman *et al.*, 2017; Voelkerding, *et al.*, 2010).

The post Tagmentation clean-up (to wash the adapter-tagged DNA on the eBLT) was achieved by use of stop tagment buffer (ST2), and tagment wash buffer (TWB). The 96-well PCR plate was placed at room temperature for 2 minutes, 10 µl ST2 added to the tagmentation solution; plate covered and incubated at room temperature for 5 minutes, positioned on a magnetic stand for 3 minutes (to dry the fluid), 60 µl of plate mixture aspirated, removed and supernatant discarded; 100 µl TWB added gently straight on to the beads and the above two steps repeated. The plate was removed from the magnetic stand and vortexed gently to re-suspend the beads, again placed on the magnetic stand for 3 minutes to clear the fluid (liquid). 100 µ supernatant was aspirated and discarded; 100 µl TWB gently added directly onto the beads. The processes of removing and discarding 100 µl supernatant and addition of 100 µl TWB was repeated 2 times. The beads were re-suspended by gentle votexing; plate was sealed and placed on the magnetic stand for 3 minutes in readiness for the next phase (Amplify Tagmented DNA) (Ill

umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis, *et al*., 2020; Nielsen, *et al*., 2011; Schoeman, *et al.*, 2018; Schoeman, *et al.*, 2017; Voelkerding, *et al.*, 2010).

Prior to starting the amplification of Tagmented DNA, an eBLT PCR program was saved on the thermal cycler with the appropriate number of PCR cycles and reaction volume of 50 µl. A PCR master mix was prepared as per the manual guide (a combination of EPM 23 µ and Nuclease-free water 23 µl x number of samples). The master mix was vortexed and centrifuged at 280 × g for 10 seconds, plate placed on the magnetic stand and 100 µl supernatant removed and discarded and plate removed from the magnetic stand. 40 µl of PCR master mix was added straight to the beads in each well (fresh tips for each well), mixed by gentle pipetting to re-suspend the beads completely, plate sealed and centrifuged at 280 × g for 3 seconds. The index adapter plate was centrifuged at 1000 × g for 1 minute and prepared by piercing the foil seal with a fresh pipette tip as per the numbers of samples. 10 µl pre-paired Index 1 (i7) was added to each well and mixed 10 times with a 40; the plate was sealed Microseal 'B', spun at 280 × g for 30 seconds, placed on the thermal cycler and eBLT PCR program switched on (total cycles and running time; 4 cycles and 46 minutes respectively). This phase preceded the clean-up Libraries step (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis, *et al*., 2020; Nielsen, *et al.*, 2011; Schoeman, *et al.*, 2018; Schoeman, *et al.*, 2017; Voelkerding, *et al.*, 2010).

This step employed double sided bead purification method to cleanse the amplified libraries. The beads (Agencourt AMPure XP beads) were placed at room temperature for 30 minutes, vortexed (to allow even distribution), drawn out and distributed gently each time, Resuspension buffer was (RBS) defrosted prior to starting the procedure. 96 well PCR plate was rocked (using a plate shaker) at 1800 rpm for 1 minute, placed on the magnetic stand for one 1 minute (to clear the fluid); 45 µl of supernatant moved from each well (PCR plate) to an identical (corresponding) well of a new midi plate; mixed by votexing, AMPure XP Beads inverted

several times to return to fluidic state. 77 µl nuclease-free water was added to all wells with the supernatant, followed with 88 µl AMPure XP Beads, plate was sealed and rocked (shaker) for 1 minute at1800 rpm and incubated at room temperature for 5 minutes. The plate was placed on the magnetic stand for 5 minute to ensure the liquid was clear. The AMPure XP Beads were vortexed and 20 µl pipetted and added to each well of the new midi plate. 200 µl of supernatant was aspirated from each well of the first plate into the matching well of the second one with 20 µl AMPure XP Beads. The second plate was sealed and rocked at1800 rpm for 1 minute; and the pre-enriched libraries were ready for pooling. The first plate was disposed of as per the safety measures (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis *et al.*, 2020; Nielsen, *et al.*, 2011; Schoeman ,*et al.*, 2018; Schoeman, *et al.*, 2017; Voelkerding, *et al.*, 2010).

The pooling method of Pre-Enriched Libraries was by volume (10-49ng gDNA). This involved combining DNA libraries with unique indexes into one pool of 12 libraries. All the information for the libraries indexes for pooling were recorded. The library pool plexity consisted of 12-plex, 2.5 µl with a total volume of 30 µl. This step preceded the Hybridize probe phase described as described in the guideline (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020).

The Hybridize Probes process involved binding targeted regions of the DNA with capture probes. Specific reagents were used to achieve desired outcome to include; Enrich Hyb Buffer 2 (EHB2), Enrichment probe panel (enrichment oligonucleotide of panel), Hyb Buffer 2 + IDT NXT Blockers (NHB2- precipitates and separates during storage) (blue cap), Resuspension Buffer (RSB), 96-well PCR plate was labelled, NF-HYB program saved on the thermal cycler using appropriate cycles (16) and reaction volume set at 50 µl. The reagents were added to the PCR plate as follows; Pre-enriched library pool 30 µl, NHB2(blue cap) 50 µl, enrichment probe panel 10 µl, EHB2 10 µl. All the well were mixed gently by pipetting 90 µl in each well 10

times. Plates were sealed (microseal 'B') and spun at 280xg for 30 seconds, placed on the thermal cycler and NF-HYB program switched on. Once the 16 cycles (115 minutes) were over, next step capture hybridized probe was started immediately (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis, *et al.*, 2020).

Capture Hybridized (combined/blended) probes step employed streptavidin Magnetic Beads (SMB) to capture the blended (hybridized) probes to the intended (targeted) areas of attention. All the reagents were placed at room temperature prior preparation as per the manual guide. A new midi plate was labelled as per the capture hybridized probe PCR plate. The sample plate was spun at 280xg for 30 seconds, 100 µl of sample from PCR plate aspirated and placed in the matching wells of the labelled midi plate, 250 µl SMB added to each well, mixed carefully, plate sealed and rocked at 1200rpm for 4 minutes, was positioned on the MIDI block micro heating system, capped and incubated for 15 minutes at 62°C, spun at 280xg for 30 seconds, placed on a magnetic stand for 2 minutes to dry. 350 µl of supernatant was aspirated out of each well and discarded and wash step carried out where 200 µl of preheated EEW added to all wells, sealed and rocked at 1800rpm for 4 minutes, placed in the incubator (62°C) for 5, spun slightly, placed on the magnetic stand for 2 minutes, 200 µl supernatant aspirated from each well and discarded.

The wash step was repeated twice prior to starting transfer wash phase. In the transfer wash, 200 µl of preheated enhanced enrichment wash (EEW) was added into each well, plate sealed and rocked for 4 minute (1800rpm). 200 µl of re-suspended bead solution was moved to a new midi plate, incubated at 62°C for 5 minutes; spun slightly, placed on a magnetic stand for 2 minute, 200 µl of supernatant aspirated from each well and castoff, plate was spun for 30 seconds (280xg), placed on a magnetic stand for 10 seconds, 20 µl of remaining fluid was pipetted out of each well and disposed. This transfer wash step done followed by the elution phase (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis *et al.*, 2020).

The elute step was accomplished by a mixer of enrichment elution buffer 1 (EE1: 28.5 µl) and 2N sodium hydroxide (HP3: 1.5 µl). This was prepared by combing EE1 and HP3, votexing briefly and spinning for 10 seconds at 280 x g. 23 µl of elution mix was added into each well, sealed and rocked at 1800rpm for 2 minutes, incubated at room temperature for 2 minutes, spun at 280xg for 30 seconds, placed on a magnetic stand for 2 minutes, 21 µl of supernatant transferred from midi plate to a new 96- well PCR plate. 4µl of elute target buffer 2 (ET2) was added into all wells with 21 µl of supernatant and briefly mixed by votexing; the plate sample was spun at 280 x g for 30 seconds prior to the amplification of enriched Library step (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis et *al*., 2020).

Amplification was used to magnify the augmented (enhanced) library. It involved use of enhanced PCR mix (EPM) and PCR Primer cocktail. AMP program was saved on the thermal cycler and set for 35 minutes, the reaction volume was set at 50 µl. 5 µl of PCR Primer cocktail (PPC) was added to all identified well of the PCR plate, 20 µl of enhanced PCR mix (EPM) added, plate sealed and rocked for 1 minute at 1200rpm, spun for 30 seconds (280 x g) placed on the thermal cycler and AMP program switched on for 35 minutes. The amplified enriched library was ready for a clean-up phase; the augmented enhanced libraries AMPure XP Beads were applied to clean the enriched library and eliminate undesirable yields (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis *et al.*, 2020).

Prior to starting the clean-up step, reagents preparation was carried out as per manual instructions(AMPure XP Beads and Resuspension buffer (RSB) were removed from storage of 4°C to room temperature and fresh 80% ethanol prepared). The PCR samples were spun at 280 x g for 30 seconds, 50 µl supernatant of every well transferred to a new midi plate, mixed by votexing, 45 µl of resuspended AMPure XP Beads was added to each well, plate sealed and rocked at 1800rpm for 1 minute, incubated at room temperature for 5 minutes, placed in the centrifuge and spun for at 280 x g for 1 minute. The plate samples was placed on a magnetic stand and held for 5 minutes, 95 µl of supernatant aspirated from each well and discarded. The

plate sample was placed on the magnetic stand and washed for two times; 200 µl fresh 80% EtOH was added to all wells of the plate sample, incubated for 30 seconds, gently without disturbing the beads supernatant was removed and discarded. All the remaining EtOH was aspirated from all wells and discarded. The plate was placed on a magnetic stand and left to air dry for 5 minutes, sealed and rocked at 1800rpm for 1 minute, incubated at room temperature for 5 minutes, placed in a centrifuge and spun at 280 x g for 3o seconds, moved to a magnetic stand and held for 2 minutes to ensure the fluid (liquid) was clear. 30 µl of the supernatant was transferred to corresponding the enriched libraries concentrations and quality was confirmed by running 1 µl using the Qubit dsDNA BR assay kit. Once the concentration and quality of the libraries was confirmed as satisfactory, the dilution process was initiated to prepare the libraries for denaturation and serial dilutions for final loading concentrations (10-12 Pm). The required starting libraries were quantified as a multiplexed pool making it the dilution based. This was accomplished by using resuspended buffer (RSB) and sequencing platform MiSeq system (v3 reagents). The final loading concentrations was optimized by an integrated flow cell Illumina in the MiSeq MPS platform ready for sequencing as shown in both **figures 5 & 6** workflows (Ill umina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis et *al*., 2020).

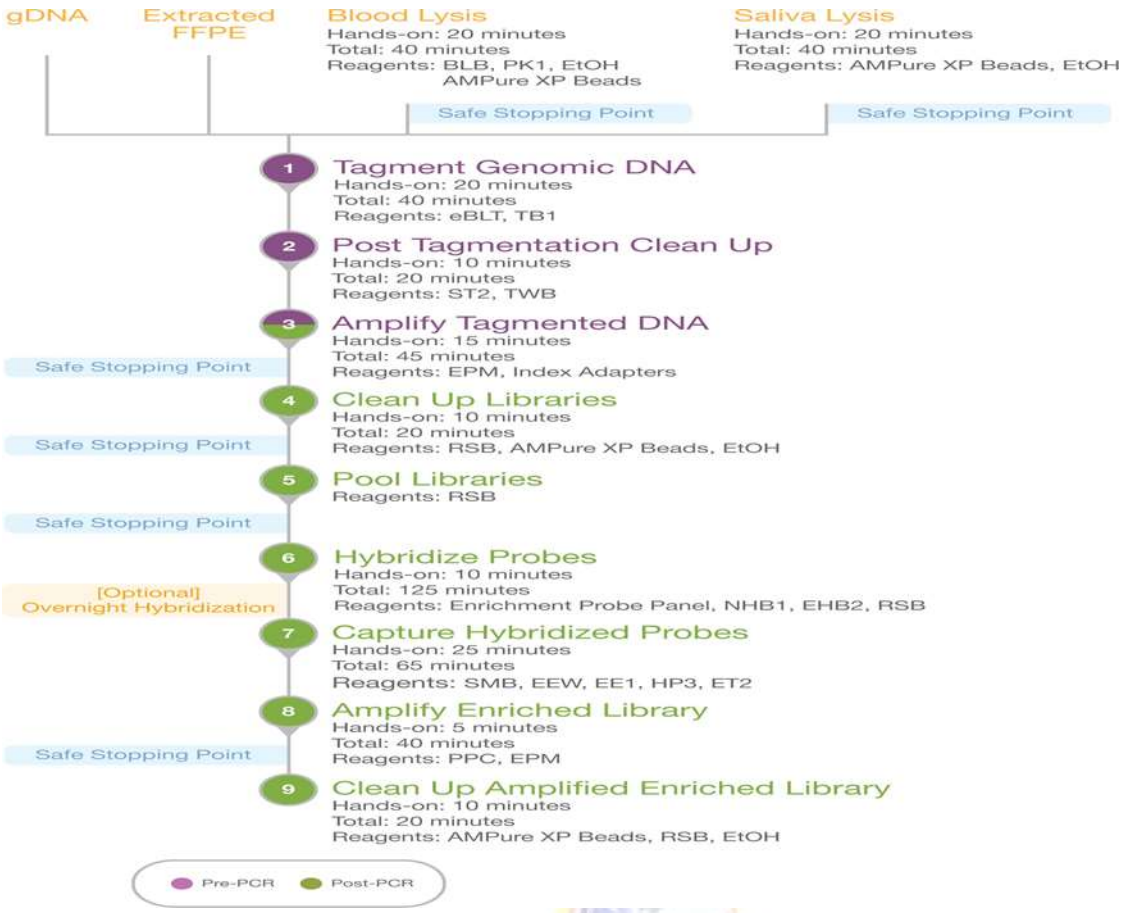

Figure 5: Illumina DNA Prep with Enrichment Workflow 1 ((Illumina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis et al., 2020)

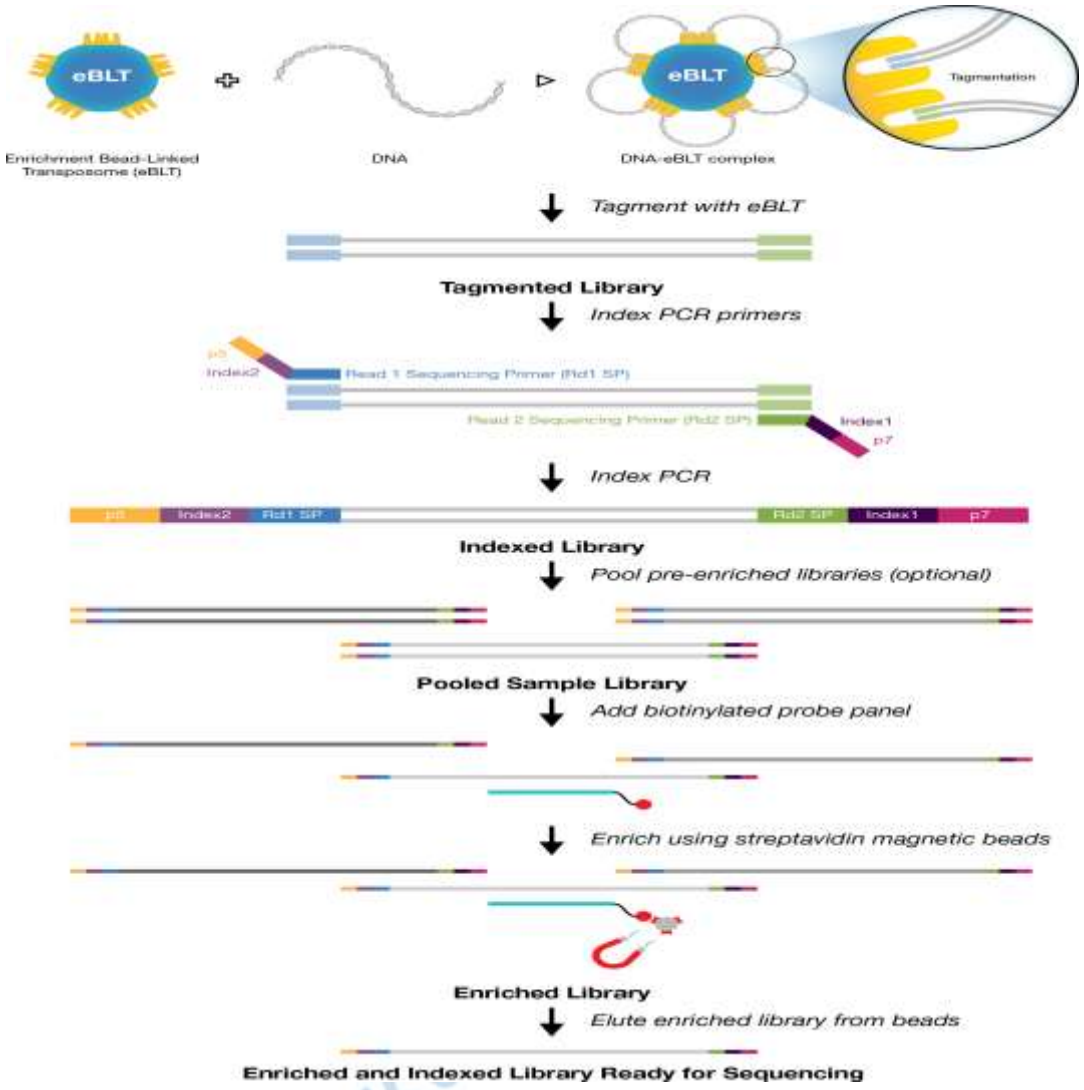

Figure 6: Illumina DNA Prep with Enrichment workflow 2 ((Illumina-Dna-Prep-Enrichment-Data-Sheet-770-2020-010.Pdf, 2020; Roulis et al., 2020)

3.8.5.4: Library Sequencing

DNA libraries sequencing was accomplished by the Illumina MiSeq platform using v3 chemistry. Variant Call Files (VCF) and Binary Alignment Maps (BAM) for each sample were generated using MiSeq reporter software according to blood group target manifest version (*MiSeq Reporter Enrichment Workflow Guide*, 2020.; *MiSeq Reporter Generate FASTQ Workflow Guide*, 2020.).

3.9: Quality Control

Quality control (QC) is a system of maintaining standards during testing or run. It ensures that the all defined steps upholds required performance standards. In this study, QC was involved in all processes from the sampling to the generation of sequencing files. The QC process was applied in all the three phases of laboratory testing to include pre-analytic, Analytic and post analytic phases. In the pre-analytic phases; the steps involved included correct identification and labelling of the samples with unique codes and appropriate samples storage. In the analytical phase, all the equipment were adjusted to correct setting specification as per the guiding procedures, upholding required temperatures (room temperature), use and constitution and storage of commercial QC materials as per manufactures instructions. This was applied during the limited automated blood group typing procedure, manual genomic DNA extraction, storage and transportation. All the reagents were accompanied with specific control materials that were either run as the test or were run prior to the test as in the case of limited automated blood grouping. The other control step was the DNA quantification that was done to ensure the DNA material was the correct volume and of integrity. During the PCR runs, all steps observed QC process as stipulated in the standard operating procedure. Before sequencing the generated libraries sizes were checked and that there were no contaminating –dimers .The QC metrics sequence analysis viewer (SAV) and illumina tool was used to monitor sequencing and also to check QC after run. The post analytical phase quality control was to interpret the results using platforms inbuilt reference materials and generated required data

3.10: Data Management

Data quality management and measurement is describes a procedure used to manage quality, validity, integrity of research data/information. This process is captured throughout the study from inception, data collection, storage, analysis and publication. The process involves five major metrics to include; Accuracy, consistency, completeness, and integrity.

In this study, data quality management was adhered to during data collection, storage, analysis and publication. The collection processes involved selection and acquisition of blood donor samples. Unique codes were generated for labelling the selected samples which were entered into an excel sheet. These unique codes were used and maintained throughout the sample handling and processing (manual genomic DNA extraction), samples shipment, quantification, sequencing, annotating, analysis, result interpretation, publication and storage. During processing, the integrity and volume of samples (4mls of blood samples collected in ETDA were assessed for consistency and conformity. Use of codes and appropriate labelling of selected samples with given codes, generating an excel sheet for the coded samples, appropriate labelling of genomic DNA, right volume (200µl) and linking them with the codes in the samples excel file. During sample shipment, an excel file was emailed to the receiving centre prior to sending the samples and was compared with the samples once received in the sequencing laboratory. After sequencing, Variant Call Format (VCF) and Binary Alignment Map (BAM) files were generated. These files contain the genomic information and thus required further management to reveal the sequence variants. The BAM files needed to be trimmed to remove data other than blood groups. This was accomplished by use of a "bam trimer" (https://github.com/MayurDivate/BamTrimmer/archive/v2.zip) to generate coverage bed files whereas the VCF required to be g-zipped which done using 7zip tool (https://www.7-zip.org/) was. This was critical to enable uploading these files to the web based system RBCeq, a combined bioinformatics webserver to distinguish blood group summaries from genomics statistics. The software used manually curated, publicly existing databank(s) to list red cell variants and described recognised, new and uncommon blood group variants. RBCeq is completely automatic and permits continuous analysis and visualization via a user-friendly interface. The generated data was exported and downloaded as an excel file for each sample. These files were checked for accuracy (ensured shipped information was similar to what was received as VCF and Bam Files), consistency, completeness (no information was missing),

integrity (data stored in the RBCEQ database accuracy and consistence and validity). Integrity/validation was managed by uploading 10% of the generated sequence files twice in the web based systems (RBCEQ) and comparing generated report for consistency and repeatability. This was also carried out during the analysis of generated downloaded report and reporting of results. Timeliness ensured that all generated data both raw and analysed is accessible in a timely manner once required (Table *et al.*, 2021. Jadhao *et al.*, 2022)

3.11: Data analysis and presentation

An excel sheet was opened and sample unique identifier codes plus study variables entered prior to processing. The information processed and control checked through all the processing. The information was transferred to Stata Version 16 for analysis for both the automated serological and sequencing data as per the objectives (appendices IV).Genomic DNA sequencing indexed reads generated FASTQ (VCF) and binary Alignment Maps (BAM) for each sample were generated using MiSeq reporter software platform and electronically transported (exported) into the CLC genomic workbench software version 8.5 (QIAGEN). Red cell variants identification was accomplished by applying adjusted Illumnia manifest file using the MiSeq inbuilt CLC genomic workbench software. The matched trimmed bam and gzipped vcf files were finally uploaded to a web based software's and aligned Wannovar and RBCeq and to the human genome reference sequence (ISBT) for variants identification. (Sudhir Jadhao *et al.*, 2022 Schoeman *et al.*, 2018; Schoeman *et al.*, 2017; Voelkerding *et al.*, 2010). https://wannovar.wglab.org/ https://www.rbceq.org/. Descriptive statistics was applied and results represented presented in tabular form. The above data analysis was applied for all the specific objectives described in section 1.5.

3.12: Ethical Considerations

The study ethical approvals were sort from Australia Red Cross and KNBTS for material transfer agreement (V). Mount Kenya University Ethical Review Committee (appendix V1

&VII), NACOSTI (appendix VIII), and Ministry of Health Kenya (IX) for authorisation to export genomic DNA materials to Australia (Queensland). The samples that were used during the study were acquired after the routine testing and to ensure confidentiality and privacy, the researcher generated unique codes that were used to for labelling and the identification of the DNAs materials. The result will be shared among the stakeholder to include, Ministry of health, KNBTS, MKU, researcher, and other health institutions to aid in decision making and for implementation consideration and further research.

CHAPTER FOUR

RESEARCH FINDINGS AND DISCUSSIONS

4.1: Introduction

This section represent the statistical information in relation to the broad and specific objectives. The subsequent analysis and explanation of data are integrated in this section of the study.

Out of 120 genomic DNA materials, 108 were fully sequenced. The researcher compared the relationship between the rbcs variants and phenotypes of the 108 sequencing data plus their distribution, frequency/prevalence patterns.

4.2: ABO Genotypes and Predicted Phenotypes Distribution

Table 6 presents the distribution of ABO genotypes and predicted phenotypes in the voluntary blood donor population. Of the 108 samples sequenced, the findings revealed ten predicated ABO phenotypes and their molecular basis. Most 52% (56/108) genotyped as O, 14%) (15/108) as B, 12% (13/108) as A1, 5.6 % (6/108) as A1/A2, 4.6% (5/108) as Ax, 4.6% (5/108) as B/B3, 2.8 % (3/108) as A2, and 1% A1B and A2B respectively. The O phenotypes molecular basis is linked to a mutation at *ABO*O.01.02/ABO*O.01.01,ABO*O.0 1.01/ABO*O.0.01,ABO*O.0105,ABO*O.01.09/A*O.01.67.*The B phenotype *ABO*B.01/AB O*O.01.01,ABO*.01/ABO*B.01,ABO*B.01/ABO*O.01.09 BO*B.01/ABO*O.01.83* molecular mutation is associated with *ABO*B.01/ABO*O.01.01,ABO*B.01/ABO*B.01AB O*B.01/ABO *O.01.09,ABO*B.01/ABO*O.01.83* allele;A1 allele is location is at *ABO*A1.0/ABO*O.01.01, ABO*A1.02/ABO*O.01.02,ABO*A1.01/ABO*A1.02,ABO*A2.06, A1/A2* mutation location at *ABO*A1.0 1/ABO*O.01.22 OR ABO*A2.01/ABO*O.01.01;A1/Ax/* Aweak Allele mutation position at *ABO*A1.01/ABO*O.01.02ORABO*AW.30.01/ABO*O.01.35,ABO*A1.02/ABO*O .01.02 OR ABO*AW.09/ABO*O.01.44,* B/B3 transformation is situated at *ABO*B.01/ABO* O.01. 02 OR ABO*B3.02/ABO*O.01.35,A2* change position is at *ABO*A2.01/ABO*O.01.29 OR AB A2.06/ABO*O.01.09,ABO*A2.01/ABO*O.01.28,ABO*A2.01/ABO*O.01.02 OR ABO *AW.09/ABO*O.01.13 OR ABO*AW. 09/ABO*O.09.02,ABO*AW.09/ABO*O.09.02* positions

the A2/Aweak phenotype mutation while *ABO*A1.02/ABO*B.01 and ABO *A2.01/ABO*B.0 1* are the molecular positions of A1B and A2B respectively.

Table 6: showing the predicated ABO phenotype and genotype Distribution (sequencing genotype data presented in this report)

System	Predicted Genotype	Predicated phenotype	n	Frequency %
ABO (n=108)	*ABO*O.01.02/ABO*O.01.01,ABO*O. 01.01/ABO*O.01.01,ABO* O.01.05, ABO*O.01.09/ABO *O.01.67*	**O**	***56***	***51.85%***
	*ABO*B.01/ABO*O.01.01, ABO*B.01 / ABO*B.01ABO*B.01/ABO* O.01.09, ABO*B.01/ABO*O.01.83*	**B**	***15***	***13.89%***
	*ABO*A1.0/ABO*O.01.01,ABO*A1. 02/ABO*O.01.02, ABO*A1.01/ABO* A1.02, ABO*A2.06,*	**A1**	***13***	***12.04%***
	*ABO*A1.01/ABO*O.01.22 OR ABO* A2. 01/ABO*O.01.01*	**A1/A2**	***6***	***5.56%***
	*ABO*A1.01/ABO*O.01.02 OR ABO* AW. 30.01/ABO*O.01.35 ABO*A1.02/ABO* O.01.02 OR ABO*AW.09/ABO*O.01.44*	**A1/Ax/Aweak**	***5***	***4.63%***
	*ABO*B.01/ABO*O.01.02 OR ABO*B3.02/ ABO*O.01.35*	**B/B3**	***5***	***4.63%***
	*ABO*A2.01/ABO*O.01.29 OR ABO*A2.0 6/ABO*O.01.09,ABO*A2.01/ABO*O.01.28*	**A2**	***3***	***2.78%***
	*ABO*A2.01/ABO*O.01.02 OR ABO*AW.9 /ABO*O.01.13 OR ABO*AW.09/ABO*O.0 9.02, ABO*AW.09/ABO*O.09.02*	**A2/Aweak**	***3***	***2.78%***
	*ABO*A1.02/ABO*B.01*	**A1B**	***1***	***0.93%***
	*ABO*A2.01/ABO*B.01*	**A2B**	***1***	***0.93%***

Legend: Distribution of the predicted ABO phenotypes established from the genotype data as presented in this report.

4.3: RhD and Weak D Frequency

Table 7 presents the incidence of D and weak D genotypes and predicted phenotypes in the voluntary blood donor's population of Kenya. Of the 108 samples the findings revealed twenty six predicted D and Weak D phenotypes and their associated genotypes mutations. The study predicted twenty six phenotypes and genotypes or alleles. The most genotyped was D at 30% followed by D-(26%), WeakD (10, 2%), Del (4.6%), D weak DDDTODV (2.8%).

RHD*Pseudogene was also revealed and was associated with a mixture D-DTO, partial and weak D, DAR3 and D-DFV types .However these RHD*Pseudogene were of low frequency ranging between 2 to 1 percent. Most of the mutation in this system were associated with gene deletion, single nucleotide variation and insertion. The predicted D positive mutation is located at *RHD*01/RHD*01 and RHD*01EL.37/RHD*03.04, RHD*01EL.37/RHD*03.09 OR RHD*01W.40/RHD*03.09 OR RHD*03.04/RHD*09.03, D negative at RHD*01N.01/ RHD*01N.01, Del at RHD*01EL.37/RHD*01 while the RHD*Pseudogene location was at different locations example DweakDDDTODV at RHD*08N.0/RHD*609G>A654G >C(M218I)667T>G (F223V)674C>T(S225F)807 T>G(Y269X).*

Table 7: Showing the Frequency of the predicted RhD & Weak D phenotypes and Variants (sequencing genotype data presented in this report)

System	Genotype	Predicated Phenotype	n	Frequency %
	*RHD*01/RHD*01*	D	32	29.63%
	*RHD*01N.01/RHD*01N.01*	D-	28	25.93%
	*RHD*01EL.37/RHD*03.04, RHD*01EL.37/RHD*03.09 OR RHD*01W.40/RHD* 03. 09 OR RHD*03.04/ RHD*09. 03,*	D	11	10.20%
	*RHD*01EL.37 / RHD*01*	Del	5	4.63%
	*RHD*08N.0/RHD*609G> A654G >C(M218I)667T>G (F223V)674C>T(S225F)807 T>G(Y269X)*	DweakDDDTODV	3	2.78%
	*RHD*01N.18/RHD*609G> A654G >C(M218I)667T> G(F223V) 674C >T(S225F)*	D	2	1.85%
	*RHD*08N.01/RHD*609G>A654G>C(M218I)667T>G(F223V) 674C>T(S225F)807T >G (Y269X)*	weakDDFV D-DTO	2	1.85%
	*RHD*08N.01/RHD*609G>A654G>C(M218I)667T>G (F223V)674C>T10(S225F) 807T> G(Y269X)*	weakDDFVDTO D-	2	1.85%

*RHD*08N.01/RHD*609G>* *A654G>C(M218I)667T>G (F223V) 674C >T(S225F) IVS3-19 dupl 37 OR RHD*609G>A654 G>C(M218I)667T>G(F223V 674C>T(S225F)807T>G (Y269X)/RHD*609G>A654 G>C(M218I) 667T>G(F223V) 674C>T (S225F)IVS3-19 dupl 37*	DTODFVweakDD	2	1.85%
*RHD*08N.01/RHD*609G>* *A654G >C(M218I)667T >G (F223V) 674C >T(S225F)* *IVS3 -19 dupl 37 OR RHD* 609G>A654G>C(M218I) 667T>G(F223V)674C>T (S225F)807T>G (Y269X)/RHD*609G>A654G> C(M218I)667T>G(F223V) 674C>T (S225F)IVS3-19 dupl 37*	DDFVDTO weakD	1	0.93%
*RHD*08N.01/RHD*609G>* *A654G>C(M218I)667T> G(F223V)674C>T(S225F) 807T>G (Y269X)*	D-DFVweakDDTO	1	0.93%
*RHD*08N.01/RHD*609G>* *A654G>C(M218I) 667T>G (F223V)674C>T (S225F)IVS 3-19 dupl 37 OR RHD* 609G> A654G>C (M218I)667T> G(F223V)674 C>T(S225F) 807T>G(Y269X)/RHD*609G >A654G>C(M218I) 667T>G(F223V)674C> T(S225F)IVS3-19 dupl 37*	DDTO DFVweakD	1	0.93%
*RHD*09.03/RHD*09.03.01* *ORRHD*09.03.01/RHD* 667T>G (F223V)819G>A*	DAR3.1 (weak Partial_ D 4.0) weak D DAR3 (weak Partial_D 4.0.1) Type 40 DFV	1	0.93%
*RHD*10.06/RHD*10.06*	DAU6	1	0.93%
*RHD*08.01/RHD*08.01*	DFV	1	0.93%

*RHD*03.01/RHD* 09.03.01*	DFV weak D D Type 40 DAR3 (weak Partial_D 4.0.1) DAR3.1 (weak Partial_D 4.0)	1	0.93%
*RHD*03.04/RHD*03.08*	DIII type 4 Partial D DNT DIII type 8	1	0.93%
*RHD*03.01/RHD*03.06*	DIII type 6 DNT Type 40 DIIIa DFV DAR3(weakPartial_D 4.0.1) DIII type 9 weak D DIII type 8 DAR3.1 (weak Partial _D 4.0) Partial D DIII type 4	1	0.93%
*RHD*03.04/RHD*03.08*	DIII type 8 Partial D DIII type 4 DNT	1	0.93%
*RHD*08N.01/RHD*609G> A654G>C(M218I)667T>G(F 223V)674C>T (S225F)IVS3-19 dupl37OR RHD*609G>A654 G>C (M218I)667T> G(F223V) 674C>T(S225F)807T>G (Y269X)/RHD*609G>A654G >C(M218I)667T>G (F223V) 674C>T(S225F) IVS3-19 dupl 37*	DTOD weak D DFV	1	0.93%
*RHD*08N.01/RHD*609G> A654G >C(M218I)667T>G (F223V)674C>T(S225F)807 T>G(Y269X)*	DTODFVweakDD-	1	0.93%
*RHD*08N.01/RHD*609G>*	DTOweakDDFV-	1	0.93%

A654G>C(M218I)667T>G			
(F223V)674C>T(S225F)807T> G(Y269X)			
*RHD*08N.01/RHD*609G> A654G>C(M218I)667T>G (F223V)674C>T(S225F)*	weakD-DFVDTO	1	0.93%
807T>G (Y269X)			
*RHD*08N.01/RHD*609G>*	weakDD-DTO DFV	1	0.93%
A654G>C(M218I)667T>G (F223V)674C>T(S225F)807 T>G(Y269X)			
*RHD*08N.01/RHD*609G>*	weakDDFVDDTO	1	0.93%
*A654G>C(M218I)667T>G(F2 23V)674C>T (S225F) IVS3-19 dupl 37 OR RHD*609G>A654 G>C(M218I) 667T>G(F223V) 674C>(S225F)807T>G (Y269X)/RHD* 609G>A654G> C(M218I) 667T>G(F223V) 674C>T(S225F) IVS3-19 dupl 37*			
*RHD*08N.01/RHD*609G> A654G>C (M218I)667T>G (F223V)674C>T(S225F)IVS*	weak D DTO DFV D	1	0.93%
3-19 dupl 37 OR RHD 609G >A654G>C(M218I) 667T> G(F223V)674C>T (S225F)807 T>G(Y269X) /RHD*609G> A654G>C(M218I) 667T>G(F223V) 674C>T (S225F) IVS 3-19 dupl 37*			
RHD			
(n=108)			

Legend: Summary of RhD and Weak D predicted phenotypes as derived from the sequencing genotype data analysis, D+ was most frequent, followed by D-, Weak D, Del and the DweakDDDTODV3 plus the weak partial D

4.4: Frequency of RHCE Phenotypes

Table 8 presents the frequency of RHCE genotypes and predicated phenotypes among the voluntary blood donor population of Kenya. Of the 108 samples sequenced the study revealed sixteen predicted phenotypes and genotypes. Most(28 %%) genotyped as c+e+, followed by V+VS+c+e+hrB+ OR C+V+VS+c+e+(18%),C+c+e+(14%), V+VS+c+ e+ hrB++OR CEST+ E-JAL+V/VS++c+e+ hrB++′-hrS++′-(12%),V+VS+c+ e+hrB+(11%), Partial_c+Partial_e+ VS+hrB+^{VW}′-OR JAL+Partial_c+Partial_e+V/VS+^{W}hrB+^{W}′-hrS+^{W}′-(7%) while the remaining 11 phenotypes had a low prevalence of between two and one percent of 2%. The mutations of this system are mostly association with insertions and structural changes located in various sites of the *RHCE* gene. The c+e+ amino acid changes are positioned at RHCE* *01.01/R HCE*01.01/RHCE*01/RHCE*01.20.10 OR RHCE*03/RHCE*01.05.01,V+VS+c+ e+hrB+OR C+V+VS+c+e+ at RHCE*03/RHCE*01.20.02ORRHCE*01/ RHCE*02.30 OR RHCE*01/RHC E*01.20.02 OR RHCE*01/RHCE*02.30,C+c+e+ at RHCE*01/RHCE*02, V+VS+c+e+hrB++ OR CEST+E-JAL+V/VS++c+e+hrB++′-hrS++′-at RHCE*01/RHCE* 01.20.01 OR RHCE*01/ RHCE* 01.20. 07, V+VS+c+e+ hrB+ at RHCE*03/RHCE*01.20.03 OR RHCE*01/RHCE*01. 20.03, Partial_cPartial_e+V+VS+hrB+^{VW}′- OR JAL+Partial_c Partial_e+V/VS+^{W} hrB+^{W}′-hrS +^{W}′-RHCE*01.20.01/RHCE*01.20.01 OR RHCE*01.20.07/ RHCE*01.20.07 OR RHCE*01. 20.01/RHCE* 01.20.01 OR RHCE*01.20.07/RHCE*01.20. 07* and the remaining being positioned in specific locations of the gene.

Table 8 : Showing the Frequency of RHCE genotypes and predicted phenotypes (genotype data presented in this report)

System	Genotypes	Predicted Phenotypes	n	Frequency %
	*RHCE*01.01/RHCE*01.01/ RHCE*01/RHCE* 01.20.10 OR RHCE* 03/RHCE* 01.05.01*	c+e+	30	27.78%
	*RHCE*03/RHCE*01.20.02ORRHCE*01 /RHCE*02.30OR RHCE*01/RHCE* 01.20.02OR RHCE*01/RHCE*02.30*	V+VS+ c+ e+ hrB+ OR C+ V+VS+ c+ e+	19	17.59%
	*RHCE*01/RHCE*02*	C+ c+ e+	15	13.89%
	*RHCE*01/RHCE*01.20.01OR RHCE*01/RHCE* 01.20. 07*	V+VS+c+ e+ hrB++OR CEST+E-JAL+V/VS+ +c+ e+ hrB++$^{w-}$ hrS++$^{w-}$	14	12.96%
	*RHCE*03/RHCE*01.20.03ORRHCE*01/RHCE* 01.20. 03*	V+VS+ c+ e+ hrB+	12	11.11%
	*RHCE*01.20.01/RHCE*01.20 .01 OR RHCE*01. 20.07/RHCE*01.20.07 OR RHCE* 01.20.01/ RHCE* 01.20.01 OR RHCE* 01.20.07/RHCE* 01.20.07*	Partial_c+ Partial_e+ V+VS+ hrB+$^{VW/-}$ OR JAL+ Partial_c+ Partial_e+ V/VS+^{W} hrB+$^{W/-}$ hrS+$^{W/-}$	7	6.48%
	*RHCE*03/RHCE*01.20.02 OR RHCE*01/ RHCE*02. 30*	V+VS+c+e+hrB+ C+	2	1.85%
	*RHCE*03/RHCE*01.06.01 OR RHCE*01/ RHCE*02*	CEAG+ c+ e+ hrB+C+	1	0.93%

*RHCE*03/RHCE*01.07.01*	CEVF+c+e+hrB+ hrS+	1	0.93%
*RHCE*01.20.02/RHCE*01.20.02ORRHCE* 02.30/ RHCE*0 2.30*	Partial_c+Partial_ e+V+VS+hrB+-OR		
	C+V+VS+ c+ e+	1	0.93%
*RHCE*01.20.01/RHCE*01.20.01 OR RHCE* 01.20*	Partial_c+ Partial_e+V+VS+hrB+		
*.07/RHCE*01. 20.07*	hrB+$^{VW/-}$ OR JAL+ Partial_c+ Partial_ e+		
	V/VS+^{W} hrB+$^{W/-}$ hrS+$^{W/}$	1	0.93%
*RHCE*03/RHCE*01.20.02ORRHCE*01/ RHCE* 02.30*	V+VS+c+e+hrB+ C+ V+VS+ c+ e+	1	0.93%
*RHCE*01/RHCE*01.08*	E+STEM+c+e+hrS +	1	0.93%
*RHCE*01/RHCE*01.20.02 OR RHCE*03/ RHCE*02.30*	E+ V+VS+ c+ e+ hrB+ OR C+ E+ V+VS+		
	c+ e+	1	0.93%
*RHCE*01/RHCE*01.20.01*	E+V+VS+c+e+hrB++	1	0.93%
*RHCE*03/RHCE*02 OR RHCE* 01/RHCE*04*	C+E+c+e+	1	0.93%
RHCE			
(n=108)			

Legend: The narrative frequency of RHCE phenotypes as concluded from the genotype and phenotypes sequence analysed data

4.5: Frequecy of MNS Phenotypes

Table 9 presents MNS predicted phenotypes and their genotypes among the voluntary blood donors of Kenya. Of the 108 samples sequenced, the study revealed four genotypes and their predicted associated phenotypes; Among the predicted types, N+s+ was the most genotyped at 40% , followed by M+M^{c}+N+s+ at 36.%, M+s+ (21.%), and M+M^{c}+N+S+ s+ at 2.8%. The molecular mutations were have also been established for all the phenotypes and were mostly associated with single nucleotide variation positioned in specific sites of the gene. N+ s+ allele site is located at *GYPA*02/GYPA*02GYPB*04/GYPB*04*, M+M^{c}+N+s+ at *GYPA* 01*08/GYPA*02; GYPB*04/GYPB*04*, M+s+ at *GYPA*01/GYPA*01;GYPB*04/G YPB*04* while the mutation responsible for M+M^{c}+N+S+ s+ is positioned at *GYPA*02/GYPA*01*08 GYPB*03/GYPB*04*.

Table 9: Showing the Frequency of MNS genotypes and the predicted phenotypes Variants (genotype data presented in this report)

System	Genotype	Predicated Phenotype	n	Frequency %
MNS(n=108)	*GYPA*02/GYPA*02GYPB *04/GYPB*04*	N+s+	43	39.81%
	*GYPA*01*08/GYPA*02; GYPB*04/GYPB*04*	M+M^{c}+N+s+	39	36.11%
	*GYPA*01/GYPA*01; GYPB*04/GYPB*04,*	M+s+	23	21.30%
	*,GYPA*02/GYPA*01*08G YPB*03/GYPB*04*	M+M^{c}+N+S+ s+	3	2.78%

Legend incidence of MNS phenotypes generated and concluded from the genotype analysed sequence data presented in this report

4.6: Frequency of Kell Phenotypes

Table 10 shows the frequency of Kell phenotypes and variants/genotypes in the voluntary blood donors of Kenya. Of the 108 samples, the finding revealed that Kell system is associated with six phenotypes and genotypes. The most genotyped was KEL2 at 79% 85 (85/108) followed by KEL2KEL6KEL7 17% (18/108), KEL1 KEL2 2% (2/108) and of the remaining 3; KEL2 KEL19, KEL2 KEL3 KEL4 KEL21, and KEL2 KEL6 KEL7 KEL19 had a frequency of one percent respectively. The mutations were responsible for the Kell phenotypes are located at specific positions of the gene; KEL2 at *KEL*02/KEL*02,* KEL2 KEL6 KEL7 at *KEL*02.06/KEL*02,* KEL1 KEL2 at *KEL*02/KEL*02.00.02,* KEL2 KEL19 at *KEL*02.-19/KEL*02*, KEL2 KEL3 KEL4 KEL21 at *KEL*02/KEL*02.21* and KEL2 KEL6 KEL7 KEL19 type location was at *KEL*02.-19/KEL*02.06*

Table 10 : Showing the frequency of Kell genotypes and predicted phenotypes (genotype data presented in this report)

System	Genotype	Predicated Phenotype	n	Frequency
	*KEL*02/KEL*02*	KEL2	85	78.7%
	*KEL*02.06/ KEL*02*	KEL2 KEL6 KEL7	18	16.67%
	KEL 02/ KEL*02.00.02*	KEL1 KEL2	2	1.85%
	*KEL*02.-19 / KEL*02*	KEL2 KEL19	1	0.93%
	*KEL*02/ KEL*02.21*	KEL2 KEL3 KEL4 KEL21	1	0.93%
	*KEL*02.-19 /KEL*02.06*	KEL2 KEL6 KEL7 KEL19	1	0.93%

KEL

(n=108)

Legend: Frequency of KEL phenotypes inferred from the genotyped analyzed data presented in this report. Most variants were found to be associated with KEL*02/KEL*02 variant at the K/k site.

4.7: Frequenncy of Kidd Phenotypes

Table 11 shows the frequency Kidd genotypes and predicted phenotypes among the voluntary blood donors of Kenya. Out of the 108 samples, the study findings show that JK was asoociated with six phenotypes/genotypes; Jk(a+b-) was the most common 28% (30/108) followed by Jk(a+b+) 24% (26/108),Jk(a+b-) 22%(24/108), Jk(a+^{W}b+) 18% (19/108), Jk(a-b+) 6.5% (7/108) and the lowest frequency recorded was Jk(a+^{W}b) *at 2% (*2/108). The mutations associated with these phenotypes are positioned in specific locations;Jk(a+b-)at *JK*01W.06/JK*01,JK*01N.20/JK*01W.06,JK*01W.04/JK*01,JK*01N.20/JK*01,JK*01W.04/JK*01W.06,*Jk (a+b+)at*JK*01/JK*02,*Jk(a+b-)at*JK*01/JK*01,*Jk (a+^{W}b+) at*JK*01N.20/JK*02,JK*01W.06/JK*02,*Jk (a-b+) at *JK*02/JK*02* and Jk (a+^{W}b) at *JK*0 1W.01/JK*01W.06*

Table 11: Showing the distribution/frequency of Kidd genotypes and predicted Kidd phenotypes (genotype data presented in this report)

System	Genotype	Predicated Phenotype	n	Frequency %
	*JK*01W.06/JK*01, JK*01N.20/JK*01W.06,JK*01W.04/JK*01, JK*01N.20/JK*01, JK*01W.04/JK*01W.06*	Jk(a+b-)	30	27.78%
	*JK*01 / JK*02*	Jk(a+b+)	26	24.07%
	*JK*01/JK*01*	Jk(a+b-)	24	22.22%
	*JK*01N.20/JK*02, JK*01W.06/JK*02*	Jk(a+^{W}b+)	19	17.6%
	*JK*02/JK*02*	Jk(a-b+)	7	6.48%
	*JK*01W.01/JK*01W.06*	Jk(a+^{W}b	2	1.85%

JK(n=108)

Legend: Summary of data for the Kidd system phenotypes inferred from sequence genotyped analyzed data showing the alleles that are responsible for the homozygosity and heterozygosity expression of Jk phenotypes (a+b-, a+b+, a+^{W}b+, a-b+ & a+^{W}b-)

4.8: Frequency of Duffy Phenotypes

Table 12 presents the the frequency of predicted duffy phenotypes and and their genetic basis among the voluntary blood donors of Kenya. Of the 108 samples, the study findings

has revealed that Duffy system has three variants and most 101(90%) are Homozygous for Fy null, Fy(a+b-) or Fy(a-b+) 4(3.7%) and Fy(a-b+) 3(2.78%). The mutation location are shown to be located at specific sites of the gene; Fy(a-b-) at *FY*02N.01/FY*02 N.01,* Fy(a+b-) or Fy(a-b+) at *FY*01/ FY*02N.01 OR FY*01N.01/ FY*02* and Fy(a-b+) at *FY*01N.01/FY*02*

Table 12: Showing the Distribution/frequency of Duffy genotypes and predicted phenotypes (genotype data presented in this report)

System	Genotype	Predicted Phenotype	n	Frequency %
	*FY*02N.01/FY*02N.01*	Fy(a-b-)	101	89.4%
	*FY*01/ FY*02N.01 OR*	Fy(a+b-) or Fy(a-b+) #		
	*FY*01N.01/ FY*02*		4	3.7%
	*FY*01N.01/FY*02*	Fy(a-b+)	3	2.78%
FY (n=108)				

Legend: # The GATA mutation on the FY gene FY c-67T>C, is more likely to be associated with the (*FY*B)* than those found to have of *FY*01(FY*A)* allele. Persons who were found to be FY*01/ FY*02 and FY c-67T>C are likely to be Fy(a+b-)

4.9: Distribution of Dantu Variants

.This study was limited in the molecular characterization of Dantu variants. Dantu system is associated three variants and it is not yet determined which one exist in the Kenyan population. However, a number of novel non-synonymous variants were identified in this genotyping dataset, which may be associated with potentially antigenic/immunogenic blood groups such as Kell and Augustine as outlined in table 13.

4.10: Frequency of Novel Variants

The study revealed 10 Novel red cell variants in different red cell gens to include; *KLF1, CD55, C4A;C4B;C4B_2, C4B;C4B_2, SLC35C1, KEL, SLC4A1, ACHE, ABCG2* and *CR1.* This was associated with the AAChange.refGene occurring in gene specific exon region as presented in t**able 13**

Table 13: Showing Novel Variant (genotype data presented in this report)

Sample	Chr	Start	Ref	Alt	Gene.refGene	AAChange. refGene
Ken 032	chr19	12996463	C	A	KLF1	KLF1:NM_006563:exon2:c.G581T:p.G194V
Ken 036	chr1	207495804	G	A	CD55	CD55:NM_000574:exon2:c.G178A:p.V60I,CD55:NM_001114752:exon2:c.G178A:p.V60I,CD55:NM_001300902:exon2:c.G178A:p.V60I,CD55:NM_001300903:exon2:c.G178A:p.V60I,CD55:NM_001300904:exon2:c.G178A:p.V60I
Ken 036	chr6	31962061	G	T	C4A;C4B;C4B_2	C4B:NM_001002029:exon20:c.G2492T:p.R831L,C4B_2:NM_001242823:exon20:c.G2492T:p.R831L,C4A:NM_001252204:exon20:c.G2492T:p.R831L,C4A:NM_007293:exon20:c.G2492T:p.R831L
Ken 036	chr6	31994799	G	T	C4B;C4B_2	C4B:NM_001002029:exon20:c.G2492T:p.R831L,C4B_2:NM_001242823:exon20:c.G2492T:p.R831L
Ken 090	chr11	45827443	G	A	SLC35C1	SLC35C1:NM_018389:exon1:c.G91A:p.E31K,SLC35C1:NM_001145265:exon2:c.G52A:p.E18K,SLC35C1:NM_001145266:exon2:c.G52A:p.E18K
Ken 096	chr7	142651330	G	C	KEL	KEL:NM_000420:exon8:c.C865G:p.L289V
Ken 107	chr17	42337835	A	G	SLC4A1	SLC4A1:NM_000342:exon6:c.T422C:p.F141S
Ken 114	chr7	100491573	G	A	ACHE	ACHE:NM_001302621:exon2:c.C281T:p.T94I,
Ken 115	chr4	89052989	G	A	ABCG2	ABCG2:NM_001257386:exon4:c.C344T:p.A115V,ABCG2:NM_001348986:exon4:c.C344T:p.A115V,ABCG2:NM_001348987:exon4:c.C344T:p.A115V,ABCG2:NM_001348989:exon4:c.C344T:p.A115V,ABCG2:NM_004827:exon4:c.C344T:p.A115V,ABCG2:NM_001348985:exon5:c.C344T:p.A115V,ABCG2:NM_001348988:exon5:c.C344T:p.A115V
Ken 117	chr1	207791445	G	A	CR1	CR1:NM_000573:exon34:c.G5569A:p.G1857R,CR1:NM_000651:exon42:c.G6919A:p.G2307R
Ken 118	chr6	44197672	A	T	SLC29A1	SLC29A1:NM_001304463:exon5:c.A469T:p.S157C,SLC29A1:NM_001304465:exon5:c.A421T:p.S141C,SLC29A1:NM_001304466:exon5:c.A418T:p.S140C,SLC29A1:NM_001078175:exon6:c.A343T:p.S115C,SLC29A1:NM_001078177:exon6:c.A343T:p.S115C,SL

	C29A1:NM_001304462:exon6:c.A580T:p.S194C

Legend: Sequencing summary showing ten novel variants of which two are associated with KEL and SLC29A1 (KELL and Augustine) blood group systems and the remaining eight are non –synonymous.

4.11: Discussion

The study broad objective was to carry out molecular characterization of red blood cell variants (ABO, D, C, E, MNS, Kell, Kidd, Duffy and Dantu) In Blood Donors at the National Testing Laboratory Nairobi– National Blood Transfusion Service -Kenya. The study sequenced a total of 108 samples applying molecular genotyping technique. The findings have revealed the predicated phenotypes and their responsible genotypes in the blood donor population of Kenya. These results reflect the present phenotypes in the Kenya population in relation to the blood systems under investigations. The choice of the specific blood groups systems is associated with their clinical significance in relation to transfusion, pregnancy, anaemia and solid organ transplantation. The current study carried out molecular characterization to determine the prevalence of red cell variants and their associated alleles in the selected population. Limited automated serology and next generation sequencing techniques were successful in predicating the phenotypes and their associated genotypes. The study outcome also elicited novel variants which had not been documented. Characterisation of Dantu was limited which was hindered by not having reference gene to align too considering it also associated with three different variants and are not yet determined in Kenya. This calls for more research in this field of science to establish a reference gene for Dantu variants.

4.11.1: ABO Genotypes Distribution

The genetic characterisation of ABO blood group system among the voluntary blood donors of Kenya showed that there are ten predicted phenotypes and their associated genotypes. This study has also established the subtypes and weak types associated with ABO. The O phenotype

was the most common at 52%, followed by B (14%), A1 (12%), A1/A2 (5.6%), Ax (4.6%), B/B3 (4.6%), A2 (2.8 %), weak A2 (2.8%) and A1B and A2B at 1% respectively. Most of the mutations associated with this system are single nucleotide variants located at specific sites of the gene. O phenotypes mutation location is at ABO**O.01.02/ABO*O.01.01,ABO*O.01.01/ABO*O.01.01,ABO*O.01.05,ABO*O.01.09/ABO*O.01.67;*B at *ABO*B.01/ABO*O.01.01, ABO*B.01/ABO*B.01ABO*B. 01/ABO*O.01.09,ABO*B.01/ABO* O.01.83* A1, A1/A2ABO*A1.01/ABO*O.01.22ORABO*A2.01/ABO*O.01.01,AxatABO*A1.01/ABO*O .01 .02 OR ABO*AW.30.01/ ABO*O.01.35 ABO*A1.02/ABO*O.01.02 OR ABO*AW.09/A BO*O.01.44 B/B3 at ABO*B.01/ABO*O.01.02 OR ABO*B3.02/ABO*O.01.35, A2 at ABO*A2.01/ABO*O.01.29ORABO*A2.06/ABO*O.01.09,ABO*A2.01/ABO*O.01.28,weak A2atABO*A2.01/ABO*O.01.02ORABO*AW.9/ ABO*O.01.13 OR ABO*AW.09/ABO*O.0 9.02, ABO *AW.09/ABO*O.09.02, A1B at ABO*A1.02/ABO*B.01 and A2B mutations locations at and ABO**A2.01/ABO*B.01*.

On comparing the findings to others from historical serological prevalence studies in O was the most common blood type O (52%). Other comparable studies conducted in Ethiopia hs shown that the O phenotype 47%, A 17%, B 14%, AB 4.8%, while genotyping frequency OO was 49%, AA-29%, AO 24%, BB 0.2%, BO 2%. Same study in Nigeria indicate has placed O frequency is at 52.93%, A22.77%, B20.64%, AB 3.66%). In Tanzania almost similar patterns is observed where O (52.3%), A (26%), B (19%) AB (3.18%) and Uganda ABO pattern also indicated O was the most common; O (50.3%); blood group A (24.6%); B (20.7%) AB (4.5%). Ethiopia, (Woldu et al., 2022 Anifowoshe A.T, et al., 2017; Githiomi, at al., 2017; Ola Jahanpou, et al., 2017; Apercu, et al., 2016).

Same studies conducted else especially in Africa show that O>A>B>AB. Considering the molecular genotyping using the next generation sequencing applied in this study, the pattern is almost similar; however most comparative studies were based on historical serology which were limited in the determination of the associated genotypes or alleles. This study outcome

further revealed the existing ABO subtypes and weak variants. The O phenotypes alleles were almost similar with most of other similar studies with minor variance regardless of the method. The A phenotype has revealed several subtypes and weak variants to include A1, A1/A2, Ax, A weak, A1B and A2B. The most common A subtype was A1>A1/A2> Ax >A2 >weak A2>. The B phenotype displayed two subtypes B > B/B3 while AB was represented by both A1B and A2B.Comparing the limited serology and genotyping outcome in this study, there was some minor discrepancy in regard to O phenotypes and A weak types where the later were mistyped as O. In this study, the findings has shown that ten samples that were serologically identified as O. However, they were of the weak A types meaning that they were mistyped as O. In regard to this mis -grouping, in case of them being patients it is an advantage for safe transfusion but as donors they were given the wrong blood group. Therefore, correct categorising the ABO genetic variations will contribute the development of algorithms alloimmunisation determination. This study new knowledge will be pivotal as a base for blood genotyping reference gene for matching red cell antigens. This study findings brings a new strategy that can be considered in regard to donor and patient blood grouping and in the investigation of alloimmunisation.

4.11.2 RhD and Weak D variants Prevalence

This is a study report on the prevalence of the predicated D variants of 108 voluntary blood donors population of the Kenya. It has been shown that weak D persons are capable of causing alloimmunisation when introduced to D− patients via transfusion, pregnancy and solid organ transplantation. Those with various D alternative phenotypes have the ability to form alloantibodies if subjected to the D+ erythrocytes or against the missing D variant (Daniels,2013). In this study molecular characterisation was employed to determine the presence of RHD red cell variants in the voluntary blood donors of Kenya. Limited automated serology was employed to determine the presence or absence of the D antigen in the blood donor samples. Most of the samples used in this study were those that were serologically

identified as D negative and weak D. The study revealed twenty six predicted phenotypes and related alleles. The most 30% genotyped was D positive followed by D-(26%), WeakD (10, 2%), Del (4.6%), and D weak DDDTODV (2.8%). RHD*Pseudogene was also revealed and was associated with a mixture D-DTO, partial and weak D, DAR3 and D-DFV types .However the RHD*Pseudogene were of low frequency ranging between 2 to 1 percent. The study went further to suggest that most of the mutations were associated with insertions/deletions, and Frameshift mutation and are located at specific sites of the gene. The predicted D positive mutation is located at RHD*01/RHD*01 and RHD*01EL.37/RHD*03.04,RHD*01EL.37/RHD*03.09 OR RHD*01W.40/RHD*03.09 OR RHD*03.04/ RHD*09.03, D negative at RHD*01N.01/RHD*01N.01,Del at RHD*01EL.37/RHD*01 while the RHD* Pseudogene site is in different locations example DweakDDDTODV at RHD*08N.0/RHD*609G>A654G>C (M218I) 667T>G (F223V) 674C>T (S225F) 807T>G (Y269X). This finding that has been generated in this report has a wealth of knowledge on the D variants in the donor population that can be used to inform on new strategies and new evidence concerning this Blood type.

Similar studies in different parts of the world has also confirmed that the frequency of D+ is most common in all the population; with approximately 85% in the white race, 95% in sub-Saharan Africa, and greater than 99·5% in eastern Asia (Daniels, 2013). Amongst the donor population of Kenya, the D frequency was found to be about 93% (Githiomi et al., 2017). The results was also compared to those of other researches carried out in other nation's world. Tunisia recorded a total of 45 weak D alleles; type 4 was the most predominant, weak D allele (1.2%), type 2 (0.17%), type 1 (0.11%) and types 5 and 11 (0.028%) correspondingly and 34 were weak D type 4 alleles (Ouchari *et al., 2015)*. The prevalence in Mali (West Africa) also show similar variants D (55%) D- (14%). DAU-0 19%, and 4% DAU-3 and other minor alleles (Wagner et al., 2003). The result is also comparable with a multinational study on RhD variants; RhD variants 11(2.2),Weak D type 4.0 4 (0.8), DV type 1 2(0.4),DAR (homozygous), 2(0.4) DAR (heterozygous) 1 (0.2), Weak D type 3.0 1 (0.2), (Wang *et al.*, 2010). Nearly all

weak and partial D variants such as DEL, DAR, and DAU6 plus others were found to be linked to the number of epitopes and insertions. In applying serology typing to identify D phenotypes, the weakly expressed types such as DEL, will be mistyped as D−. This study found that some of those that were serologically identified as D negative were Weak D positive and the weak D were D positive. From the results generated from this study, it is evident that serological determination of the D antigens is limited in the identification of the weak D variants as well the pseudogenes. The findings of this study has shown that most of the D variants cannot be identified by haemagglutination or serological methods including antiglobulin (indirect antiglobulin) tests. This research therefore brings the required evidence that needs to be considered in the determination the weak D variants particularly to those who receive continuous blood transfusion therapy. Nevertheless, caution ought to be taken to ensure that weak D antigen testing is carried out in well-coordinated environment to ensure false positive are eliminated to avert unintentional transfusion of Rh D- erythrocytes to Rh D-negative cases with serious immunological and clinical reactions. This gap can be addressed by application of molecular genotyping and ensuring there are appropriate blood management and transfusion guidelines specific for each country. Most appraised studies except some few used serological method in the determination of D antigen prevalence and thus are limited in terms of revealing molecular basis or genotypes/alleles that are responsible for the of D variants. The current study applying red cell genotyping has revealed the molecular basis of the RHD variants. This report is a plus towards the strengthening the alloimmunisation investigations as well developing new guidelines or review the existing ones in regard to this blood group in relation to mothers of child bearing age as well introducing new testing strategies.

4.11.3 RHCE Phenotype (Rare red cell variants)

This discussion is based on the study report on the prevalence of the predicated of the RHCE variants of 108 voluntary blood donors population of the Kenya. The RHCE red cell variants are considered to be the rare types. The study evaluated the encoding gene in order to predict

the present phenotypes and genotypes plus their specific locations. The study employed genotyping technique (Next-generation sequencing) in their determination). It is important to confirm that the presented data was pull out from the D variants one considering that both are in the same Blood Group System.

This study findings; RHCE predicated phenotype was guided and inferred from the sequenced genotype data. The findings revealed a combination of sixteen predicted phenotypes and associated genotypes; the most genotyped was c+e+ found at 28% followed by V+VS+ c+e+ hrB+ OR C+V+VS+c+e+17.6%,C+c+e+at 13.%,V+VS+c+e+hrB+at11.1%),Partial_c Partial_e+V+VS+hrB+^{VW}⁄- OR JAL+Partial_c Partial_e+ V/VS+^{W} hrB+^{W}⁄- hrS+^{W}⁄- found to be 6.5% while the remaining proteins frequencies at this site ranged between 2 to 1 percent. There was no serological data locally available to compare the study outcome. The hrB+ has displayed a weak association with the c+e+, this could be attributed to insertions.

On comparing the study results with other historical similar ones there is some level concurrence with minor variation being observed in different populace? Research from Saudi Arabia, indicated that the e phenotype was the most frequent (97%), and c (86%) (Amani *et al*., 2020). In North India e was displayed as the most predominant (98.3%), C (84.76%), c: (52.82%) and E(17.9%) *(Thakral et al., 2010*). In South Africa black population, ce/ce: 42% *(*Govender *et al., 2021*). A study carried out in America among the American black indicated that 1 out of 50 was found to have hrS and hrB (Westhoff et al., 2013). The study further indicated that they were associated with weak c, e phenotypes. These findings of Saudi Arabia, North India and South Africa have shown that the c,e phenotypes are the most common and this is also observed in the outcome of this study. The only variance that is observed is in percentages. Therefore, the c,e antigens are of high prevalence in these populations and thus not likely to cause alloimmunisation unless introduced to those who lack these specific immunogens. The other type of phenotypes that are linked to this system and were revealed in this study are VS and hrB. The study has further shown that they are associated with weak c,

and e. This has further revealed that when VS are homozygous they are hrB negative These antigens have also been reported in similar studies as reported to be of low prevalence ((Sippert et al., 2021. Westhoff et al., 2013). However, in this study their prevalence range is between 18 to 1 percent meaning that it of clinical significance in the field of transfusion and transplantation. This because they are capable of alloimmunisation when introduced to those who lack them resulting to nasty haemolytic transfusion reactions

The outcome study has also established that blood groups genotyping can be used in resolving complicated cases related to RHCE alloimmunisation. VS and hrB have been identified as the most common phenotypes in Africans and of clinical importance in terms of isoimmunization, therefore a well detailed guideline on how to identify the RHCE phenotypes in donors and patients and also in the management of those identified to be immunised against them is critical. A comparative study on hrB, VS and JAL phenotypes in relation to other Sub-Sharan African Countries is required to establish their prevalence and association with haemolytic transfusion reactions.

4.11.4 MNS Phenotype Frequency (Rare red cell variants)

Landsteiner and Philip Levine described the MNS in 1927. It is very polymorphic next to ABO and RhD with 50 described antigens. It is composed of two glycophorin GPA and GPB located on the red cell membrane with four phenotypes; M,N,S and s (Faria et al., 2012). MNS antigens occur due to numerous genetic alterations with the major one being associated with Single nucleotide variations (SNVs) resulting to the enormous variety of the antigens in this blood group system. M and N phenotypes are common across many populations and also genotypes The S–s–U– phenotype is uncommon in Asians and white populations nevertheless it is more common in the Africans. S, s and U are of clinical significance in terms of pregnancy and transfusion. If introduced to those who lack this antigen, they are capable of triggering an immunological response in form of antobdies production that have been associated in both

serious and after transfusion responses and also in Hemolytic Diseases of the fetus and new born (Faria et al., 2012; Lopez et al., 2021)

MNS phenotypes from the genotype data revealed four phenotypes ; N+s+ found at 40% was the most common genotype, followed by M+M^{c}+N+s+ at 36.1%, M + s+(21.3%), and M+M^{c}+N+S+s+ reporting a frequency of 2.8%. There was no serological data locally available to compare the study outcome. The generated data/results display some minor variation in comparison with reports of similar studies conducted in different populations globally.

Dean, 2005, documented the occurrence of MNS phenotypes: M+N+s+: 22% whites, 33% black colour, N+S+s+:24% whites, 13% black colour M+S+s+:14% whites, 7% Black colour. Lowest prevalence was recorded in M+N+S+ 4% white ,2% Black colour) and N+S+ (1%whites ,2% Black colour).The phenotypes M+,M+N+ and N+ are infrequent in most population with ~0.5% in the people of black colour (Lopez *et al.,* 2021). The Kenyan MNS data has revealed a low frequency antigen M^{c}+which can result to alloimmunisation if exposed to those who are negative.

In India, the MNS phenotype frequencies; M+N+ 54.15, per cent in Indians, 50 % in whites 44 % in colored (Blacks) race 47.1% in the China community, S(54.8%) and s(88.7%), s+ type was the most occurring phenotype in Indian and whites populations as in Caucasians (45%) and Blacks (69%) respectively (Lopez *et al*., 2021). In Saudi Arabia, M+N-S+s+ and M+N+S-s+, genotyped as 24% and 21% correspondingly. However, N+S+s+ and M-N+S+s- showed a low frequency of 3% demonstrating variation with other studies conducted in other places globally (Lopez *et al*., 2021). In North India, recorded phenotypes were M+N+S+s+ (19.55%) and M−N+S+s− (1.26%) (Lopez *et al*., 2021).

The study findings among the blood donor population of Kenya show that N and s combination at 40% to be the most frequent among the blood donor population of Kenya. Considering N to be common in the populations when combined with s there is a risk of alloimunisation. When s is introduced to those lacking via transfusion and pregnancy, anti-s

will be formed and since it is of IgG type, it will result to advers transfusion reactions and HDFN. This study findings presents evidence that can be used to form testing guidelines in regards to the identification of MNS phenotypes both in blood donors and recipients plus expectant mothers. They will also form the basis of reviewing existing blood transfusion guidelines and consider reviweing and developing testing guidelines to aid in the identification of the MNS phenotypes. The outcome has establihsed that blood groups genotyping can be used in resolving complicated cases realted to MNS alloimunistaion.

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(Lopez *et al*., 2021). North India has recorded two phenotypes comprising; M+N+S+s+ (19.55%) and M−N+S+s− (1.26%) (Lopez *et al*., 2021)

4.11.5 Kell Phenotype and Genotypes (Rare red cell variants)

The Kell system is 006 in the ISBT list. It is also among the most important blood groups in clinical significance in relation to transfusion and obstetrics medical conditions. It was recognised in 1946 and its antigen and associated alleles/variants was later revealed in 1449 by Levine and colleagues. It is highly immunogenic and is also linked to adverse effects in transfusion and pregnancy. This blood group system is system is associated with 36 antigens. A series of the Kell antigens are expressed antithetically with a mixture of high and low occurring types to include, KEL2 (k)/KEL1 (K), KEL4 (Kpb)/KEL3 (Kpa)/KEL21 (Kpc), KEL7 (Jsb)/KEL6 (Jsa), KEL11/KEL17, and KEL14/KEL24.

This study molecular characterisation of Kell revealed that Kell system has six variants and their predicted phenotypes. The most common was KEL2 (KEL*02/KEL*02) 85(79%) followed by KEL2 KEL6 KEL7 (KEL*02.06/KEL*02) 18 (16.7%). The rest were found to be of low frequency and all were associated with KEL2. The predicated phenotypes inferred from the genotype data presented predicted that the k (KEL2) phenotype was the most common while variants at other locations in the KEL protein were most frequently associated with the KEL*02/KEL*02 variant at the K/k site. There was no serological data locally available to compare the study outcome.

The study outcome is slightly different from what was observed by Dean 2005, KEL2 (k+) was 91% in the white population, and 98% in the people of black colour. KEL1 (K) was recorded as having a low frequency of 0.2% in whites and very rare in the dark population. KEL1/KEL2 (K+k+) 8.8% in whites and 2% in people of black colour (Dean, 2005).

In the Chinese community, K (KEL1) frequency was 3.5% and k (KEL2) 99.97% (Liu, et al., 2012). In Indian study, KEL2 (k+) (96.5%), (91%) in whites and (98%) dark colour (blacks) and 100% in Chinese populace (Makroo, et al., 2013). In Northern India the incidence of Kell

types have been shown as KEL1 (K) 2.57% whereas KEL2 (k+) as 97.43% (Mwangana, *et al*., 2021; Thakral, et al., 2010). In the Japanese population the study publicised that KEL*01/KEL*02 as (0.48%); KEL02/KEL02 (99.52 %.) KEL*02 was 99.76% and KEL*01.1 (0.024%) (Flôres, *et al*., 2013). Study from Saudi Arabia indicates that KEL2 (k+ Cellano) was the most common (100%) whereas KEL1 (K) recorded 8% (Amani, *et al*., 2020).A study from Morocco, KEL1 (K) (0.19%) whereas KEL2 (99.80%) (Benahadi, *et al.*, 2014) .Similar study conducted in Southern Brazil documented the frequencies as; KEL*2/KEL*2: 94(75%), KEL*1/KEL*2: 5.0% while KEL*1/KEL*1 was 0.25% (Flôres, et al., 2014).

The molecular characterization carried out for the Kell red cell variants have elucidated the genetic foundation of Kell phenotypes. Most of the variants are due to single nucleotide mutation. KEL2(k+) was the most common at 77%, followed by combinations of K(KEL2) indicating that it is of high frequency in the donor populations of Kenya. Kell (K, Jsa and Jsb) recorded a 15.4%. The k+ Jsa and Jsb antigens are of medical significance in transfusion, pregnancy plus solid organ transplantation. Kell system alloimmunisation has been incriminated in influencing against HLA antigens in solid organ transplants (Holt et al., 2020). Having sensitive method such as red cell phenotyping and molecular genotyping of blood groups will be key in the identification of these phenotypes to reduce associated alloimmunisation. Therefore, this study outcome has generated sufficient molecular evidence that can inform on reviewing and development specific testing guidelines.

4.11.6 Kidd Phenotype Frequency (Rare red cell variants)

The Kidd blood system was described in 1951. It has twofold major phenotypes Jka and Jkb. It is of medical importance in transfusion therapy, obstetrics plus transplantation. The antigens can be identified using serology and molecular applications. In this study, molecular genotyping was applied to identify their molecular basis and associated phenotypes. The outcome revealed six Kidd phenotypes to including; heterozygosity expression of Jk (a+b-) 28%, homozygous for Jk (a+b+) 24.1%, homozygous Jk(a+b+) 22.22%), heterozygous for Jk

(a+^{W}b+) 18%, homozygous for Jk (a-b+) 6.5% and heterozygous Jk (a+^{W}b-) at 2%. The null phenotype devoid of JK (a-b-) was not detected in this study. There was no serological data to compare the findings. Kidd system phenotypes inferred from genotype data presented indicated. This study outcome was also comparable with other similar studies carried out in different populations. A study by Dean 2005, indicated that there is variance in the frequency of JK phenotypes across different populaces; Jk(a+b+): was 50% whites, 41% Dark colour, 49% Asians; Jk(a+b-): 26%, whites 51% dark colour ,23% Asians, Jk(a-b+): 23% whites , 8% dark people, 27% Asians and JK(a-b-) was of very low prevalence and was only recorded in Polynesians 0.9% (Dean, 2005).

In south Brazil, the most occurring variant was JK*A/JK*B (48.0%), JK*A/JK*A (27.25%) and low prevalence was recorded as JK*B/JK*B (24.75%) (Guelsin et al., 2011). Among the Chinese (Jiangsu) population, the recorded JK phenotypes frequency was Jk*01(Jk(a+)(51%),and Jk*02(Jk(a-b+)) 49% (Liu, *et al*., 2012).

A study amongst blood donors of Morocco revealed that Jka was 84.21%, Jkb 65.3%, Jk (a+b+) 49.51%, and Jk (a−b+) 15.79% (Benahadi *et al*., 2014). Among the Indians blood donors frequent occurring JK phenotypes was Jka (81.4%) , Jkb (67.6%), and Jk (a+b+) 57% (Makroo, *et al.,*2013*)*.

The Kidd genotypes frequencies among the Brazilian Japanese population showed the following recording; JK*01/JK*01(24.40%); JK*01/JK*02 (43.54%); JK*02/JK*02(32.05%), JK*01 (46.17%); and (JK*02 53.83%) (Flôres *et al*., 2014).

A case study review by Sanford and colleagues observed that Jka was the most prevent in the world population with 90% in African Americans, 77% in Caucasians, and 73% of indigenous Asians. Jkb exhibits a related occurrence amongst Caucasians and ethnic Asians with a low occurrence in African Americans (~49%). The Jka-b-and Jk3 were the least common among the JK phenotypes (Sanford *et al*., 2015).

The JK molecular characterisation data shows that Jka is the most common phenotype among the blood donor population of Kenya 28% compared with Jkb at 6.5%. Jkab was 22.2%.In most of the cases, Jkb has been shown to be in combination of Jka/ Jka+^{W}.The other phenotype that was revealed with a notable frequency was and is not common comparing with other studies is Jk (a+^{W}b+) 18%. It is also worth noting that this phenotype is mostly associated with Jka. The Kidd phenotypes are of clinical significance in transfusion and pregnancy and thus they should be determined in donors to ensure there is matched units given for those receiving ongoing transfusions. This requires documented blood management guidelines and testing protocols (phenotyping and genotyping protocols).

4.11.7 Duffy Phenotype Frequency (Rare red cell variants)

Duffy Blood Group System was described in 1850 by Marie Cutbush and Patrick Morison. The Duffy antigen is also called FY glycoprotein. It has two major antigens Fya and Fyb. Fya and Fyb phenotypes are immunogenic and are capable of causing immunization with Anti-Fya and Fyb immunoglobulins. The Fy antibodies are of medical importance in pregnancy and transfusion therapy (Dean, 2005). The Duffy phenotype is a chemokine receptor (DARC) and is linked with invasion of young red cells (reticulocytes) by a type of malaria parasite known as Plasmodium vivax. Studies conducted among the African population identified the null type Fy(a-b-) as the most frequent; meaning most of the people are protected from this type of malaria infection that is associated with Plasmodium vivax (Rodriguez, 2020).

The Molecular Charactrization of the Duffy red cell varinats revealed that three phenotypes are associated with that the this system among the Kenya blood donor population ; homozygous for FY*Null GATA regulatory box variant *FY*02N.01/FY*02N.01* or Fy(a–b–) found in 93.52% (101/108), while *FY*01/FY*02* and FY c.–67T>C predicted Fy(a+b–) or Fy(a–b+) at 3.70% (4/108) while *FY*02/FY*02N.01* predicted Fy(a–b+) at 2.78% (3/108). The most common phenotype was Fy(a–b–) found in 93.52% showing that Fyab- is the prevalent

phenotype in the population.This outcome is of importance considering the fact that it is protective against the malaria caused by Plasmodium vivax (Rodriguez, 2020).

Comparing the results with other studies in different populations there are some minor variations that were observed. In South Africa black population Fy(a-b-) 39% while Fya+ Fyb+ was reported to be 2% (Govender *et al*., 2021). In Saudi Arabia, type Fy(a-b-) was identified in 11.11% (Benahadi *et al*., 2014). In the Brazilian Japanese population, FY*01/FY*01(Fya+) occurrence was 59.33%, FY*01/FY*02 (Fya+ Fyb+) 38.27% and FY*02/FY*02 (Fyb+) was 2.39%. In sub-Saharan African people, FY null phenotypes frequency have been shown to be ≥95% (Flôres, et al., 2014; Höher, *et al*., 2018). In the Indian race study Fy (a+b-) was most frequent 42.1%, Fy (a+b-) was most occurring type in the white populace 49%, and Fy (a-b-) most prevalent in the coloured people 68% (Howes, *et al*., 2011). Most of those conveying the null phenotypes were from the black population (Makroo, *et al.*, 2013). A study championed by Carlos Rodriguez in 2020, revealed that most Africans have FY (ab-) that is the FY null phenotype. He also observed that 68% of the African Americans and 88-100% and 90% people of West Africa were Duffy negative Fy(ab-). The null FY (ab-) type silences the gene in the erythroid cells. He together with other authors have shown that this null Duffy phenotype is rare in the Caucasians (Rodriguez, 2020).

The molecular characterization of Duffy phenotypes in Kenya has signified that there are Fy phenotypes and genotypes associated with this system among the Kenya blood donor population.They include the FY*Null GATA regulatory box variant *FY*02N.01/FY*02N.01* or Fy(a–b–); this was found in found in 101/108 (93.52%), followed by a heterozygous type *FY*01/FY*02* and FY c.–67T>C that predicted Fy(a+b–) or Fy(a–b+) at 3.70% (4/108) while *FY*02/FY*02N.01* predicted Fy(a–b+) at 2.78% (3/108).

In most of the studies conducted among different nations of the world, the FY null (Fyab-) phenotype has been shown to be the most common type as well as in the black people. In most of the cases, the FY null (Fyab-) could be equated to protective genetic mutation against malaria

invasion. Considering their clinical significance in relation to delayed transfusion reactions and HDFN. They have are associated with alloimmunisation once introduced to those who lack that lack them. Considering the three genotyped variants in this study, an essential rare genotyped pool of donors can be identified in support of the provision of matched red cell units when required. It is also important to have blood management guidelines on how these FY phenotypes will be identified in all donations and screened in patients who are continually transfused. Extended serological typing using specific red cell and antibody panels should be included in the blood banks testing protocols to ensure these FY phenotypic antigens are not missed. Genotyping should also considered to resolve complicated cases associated with FY phenotypes.

4.11.8 Frequency of Dantu Variants

Dantu variants are uncommon in most populations' worldwide (Palacajornsuk,2006*)* (Palacajornsuk, 2006). Dantu variant have is associated with three types variants and it has not been established which of the three variants is in Kenya. Therefore, due to the limitation of knowing which Dantu variants are frequent in Kenya, this study was limited in the characterization of Dantu variants because there was no reference gene to align these variants; not disregarding that this is a very phenotype among the world populations (*Moghaddam & Naghi, 2018)*. However, this study drawback in the characterisation of Dantu variants does not limit more research in the field to determine the prevalence of data with more data regarding this phenotype. Considering that it is a rare variant in the world population, documentation of its prevalence is essential as well as learning more of associated clinical significance in depth.

4.11.9: Frequency of Novel Variants

This study revealed a number of novel non-synonymous variants were identified and confirmed by ID numbers and copy variant (Amino Acid) change. However, it is worth noting that some of these revealed new variants may be potentially antigenic/immunogenic blood groups such as Kell and Augustine. Kell antigens are associated with HDFN and thrombocytopenia or

decreased fatal thrombocytes counts while Augustine antigens have been incriminated to be major causes of haemolytic diseases of the fetus and new born plus adverse transfusion reactions. (*Moghaddam & Naghi, 2018)*.

Augustine (AUG) blood group system was discovered in 1967 in an African American Woman through her third gestation. After this discovery of 1967, more revealed were documented in persons of African American Race. Augustine (AUG) blood group system has four antigens: AUG1, AUG2 (Ata), and AUG4 to be the most common in the population. However the fourth antigen AUG3 records a low prevalence compared with the other three. The antigens associated with the AUG location is found on ENT1 which equilibrize nucleoside transporter coded by SLC19A1. ENT1 is found in nearly all human tissues. It has a major function of aiding the transferring purine and pyrimidine amino acids plus they are accountable for transporting numerous adenosine through plasma membranes. Adenosine carriage has an important influence in the regulation of bone metabolism. AUG antibodies are of scientific significance in blood transfusion and pregnancy. Anti-AUG2 have been linked to haemolytic transfusion responses; while anti-AUG3 has been associated with causing serious haemolytic disease of the fetus and new-born (Daniels, 2021). The AUG phenotypes need to be identified both in donors and patients to reduce alloimmunisation. There is need to have robust documented procedures and guidelines entailing how these phenotypes will be identified.

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1: Introduction

This section entails the study summaries, conclusions and propositions resulting from the conduct of the research. This study was conducted at national Testing laboratory-KNBTS (-1.29965, 36.81184); Sampling and Limited ABOD automated serology testing site. Manual DNA Extraction, purification and storage was carried out at National Malaria Public Health Laboratory-Kenya (-1.30412, 36.80707) prior to shipment to Australia and Australia Red Cross Life blood laboratory (-27.45007, 153.01267) for quantification and sequencing. The target subjects were voluntary blood donor's samples drawn in EDTA tubes that were used for routine blood grouping purposes. Purposeful sampling, cross sectional, and experimental design were employed during the study. The raw data was obtained via next generation sequencing (NGS) performed on 12-plex pools using the Illumina MiSeq with standard 300 cycle V2. Limited serological automation was also applied prior to determine the ABO, D phenotypes in the selected samples prior to the manual DNA extraction and purification procedures. Data management was accomplished by acquiring the Genomic DNA sequencing indexed reads generated FASTQ (VCF) and binary Alignment Maps (BAM) for each sample that were generated using MiSeq reporter software platform and electronically transported (exported) into the CLC genomic workbench software version 8.5 (QIAGEN). Red cell variants identification was accomplished by applying adjusted Illumnia manifest file using the MiSeq inbuilt CLC genomic workbench software. The bam and vcf files were finally uploaded to a web based software's Wannovar and RBCeq and aligned and to the human genome reference sequence (ISBT) for variants identification. The software generated excel files that were downloaded and stored. The automated serology results were generated by the neo immucor platform and were downloaded and recorded. The downloaded raw data was uploaded to Stata version 16 statistical tool which was used to analysis generate data in form of value and

percentages. The ethical approval for the study was sort from Mount Kenya University, Ministry of Health, and NACOSTI and for sample shipment from PPB Kenya. The other collaborative approvals were sort from Australia Red Cross Lifeblood.

5.2 Summary of Study Findings

The main objective was to carry out Molecular Characterization of Red Blood Cell Variants (ABO, D, C, E, MNS, Kell, Kidd, Duffy and Dantu) In Blood Donors at The National Testing Laboratory –Kenya Blood Transfusion Service. The outcome of the study have revealed the molecular basis of six (ABO, RHDCE, MNS, KELL, KIDD and DUFFY) Blood Group Systems among the seven listed in the main objective. There was limitation in revealing the molecular basis of Dantu variants due to lack of a reference gene to align these variants. The findings of this study is presented applying the specific objectives as outlined below

ABO Genotypes Distribution: ABO predicted phenotypes and Genotypes Distribution: This study findings revealed the genotypes that were responsible for the ABO phenotypes. The O phenotype at (52%) (56/108), was the most common, followed by B 14%(15/108), A1 at 12% (13/108), A1/A2 at 5.6% (6/108),Ax at 4.6.% (5/108), B/B3: 4.6%,(5/108) A2:2.8 %, (3/108),weak A2:2.8% (3/108)and whereas A1B and A2B at of 1% respectively. Most of the ABO mutations were associated with a single nucleotide variation:

RhD and Weak D Variants Frequency: The study predicted twenty six phenotypes and genotypes or alleles. The most genotyped was D at 30% followed by D-(26%), WeakD (10, 2%), Del (4.6%), and D weak DDDTODV (2.8%). RHD*Pseudogene was also revealed and was associated with a mixture D-DTO, partial and weak D, DAR3 and D-DFV types .However these RHD*Pseudogene were of low frequency ranging between 2 to 1 percent. Most of the mutation in this system were associated with gene deletion, single nucleotide variation and insertion. The predicted D positive mutation is located at RHD*01/RHD*01 and RHD*01EL.37/RHD*03.04,RHD*01EL.37/RHD*03.09 OR RHD*01W.40/RHD*03.09 OR RHD*03.04/RHD*09.03,Dnegative at RHD*01N.01/RHD*01N.01,Del at RHD*01EL3 7/R HD*01while the RHD*Pseudogene location was at different locations example D weakD

DTODV at RHD*08N.0/RHD*609G>A654G>C (M218I) 667T>G (F223V) 674C>T (S225F) 807T>G (Y269X).

RHCE Prevalence: In this study, the predicted of the RHCE phenotypes and their linked alleles employed next generation sequencing. The results elucidated seventeen phenotypes and genotypes. There was no serological determination of these antigens. Among the revealed predictions, c+e+ was the most common at 28%, followed by V+VS+c+e+hrB +OR C+V+VS+c+e+-, (18%), C+c+ e+(14%)V+VS+c+e+hrB ++ OR CEST+JAL+V/VS++ c+e+ hrB++′-hrS++′- (13%), V+VS+ c+ e+ hrB+ (12%), Partial_c+ Partial_e+ V+VS+ hrB+^{VW}′- OR JAL+ Partial_c+ Partial_e+ V/VS+^{W} hrB+^{W}′- hrS+^{W}′- (6.5%) and the remaining 11 types had a frequency range of between two and one percent . The study outcomes suggests that most of the system polymorphism are associated one nucleotide alteration and, frame makeshift and insertions. Further to the phenotypes predictions, the results have also shown the sites of the mutation or allele positions in the gene. The c+e+ mutation or transformation is located at RHCE*01.01/RHCE*01.01/RHCE*01/RHCE*01.20.10 OR RHCE*03/RHCE* 01.05.01,V+VS+c+e+hrB+at RHCE*03/RHCE*01.20.02 OR RHCE*01/RH CE*02.30 OR R H CE*01 /RHCE*01.20.02OR RHCE*01/RHCE*02.30, C+c+e+ at RHCE*01/RHCE*02,C EST+AL+V/VS++c+e+hrB++′-hrS++′- at RHCE*01/RHCE*01.20.01 OR RHCE*01/RHC E*01.20.07, V+VS+ c+ e+ hrB+ at RHCE*03/RHCE*01.20.03ORRHCE*01/RHCE* 01.20. 03, Partial_c+ Partial_e+ V+VS+ hrB+^{VW}′- OR JAL+ Partial_c+ Partial_e+ V/VS+^{W} hrB+^{W}′- hrS+^{W}′- at RHCE*01.20.01/RHCE*01.20 .01 OR RHCE*01. 20.07/RHC E*01.20.07 OR RHCE* 01.20.01/ RHCE* 01.20.01 OR RHCE* 01.20.07/RHCE* 01.20.07 and the remaining were phenotypes had their mutations location at specific sites in the RHCE gene.

MNS Prevalence: The study predicated four red cell phenotypes and responsible genotypes. N+s+ was the most frequent(39.8%), M+M^{c}+N+s+ (36.1%),M+s+,(21.3%), and M+M^{c}+N+ S+s+ (2.8%). Most of the predicted alleles were due to single nucleotide variations. N+s+

genetic mutation location was at GYPA*02/GYPA*02GYPB*04/GYPB*04,M+M^{c}+N+s+ at GYPA*01*08/GYPA*02,GYPB*04/GYPB*04,M+s+atGYPA*01/GYPA*01,GYPB*04/GYPB*04 while M+M^{c}+N+S+s+ phenotype mutation position is at GYPA*02/GYPA*01*08 GYPB*03/GYPB*04.

Kell (KEL) Prevalence: The study established that Kell system has six variants with two major phenotypes and genotypes. The most common phenotype was KEL2 at 79% (85/108), followed by KEL2 KEL6 KEL7 at 16.7% (18/108).The rest were found to be of low frequency and all were associated with KEL2**.** The mutations were associated with single nucleotide alterations located at different site of the KELL gene: KEL2 mutation location was at KEL**02/KEL*02,* KEL2 KEL6 KEL7 at *KEL*02.06/KEL*02* and the four remaining phenotypes mutations at different positions along the *KEL* gene. The nucleotide variations are located in different sites of the gene including; KEL2 at *KEL*02/KEL*02*, KEL2 KEL6 KEL7 at *KEL*02.06/KEL*02,* KEL1 KEL2 at *KEL*02/ KEL*02.00.02,* KEL2 KEL19 at *KEL*02.-19/KEL*02,* KEL3 KEL4 KEL21 at *KEL*02/KEL*02.21/KEL2* and KEL2 KEL6 KEL7 KEL19 at *KEL*02.-19 /KEL*02.06.*

Kidd (JK) Prevalence: The outcome revealed six red cell variants and phenotypes associated with Kidd (JK) system. The most predominant type was Jk (a+b-) 27.8%, (Jk (a+b+) 24.1%, Jk (a+b) 22.2%, (Jk (a+^{W}b+) 17.6%, (Jk (a-b+) 6.5%, and Jk (a+^{W}b-) 2.0%. The null JK (a-b-) phenotype was not detected in this study. This study has established the molecular basis location was mostly due to one nucleotide alterations. This study further revealed the location or site mutations of the genotypes or alleles that are responsible for the predicted phenotypes. Jk(a+b-) allele is positioned at *JK*01W.06/JK* 01,JK*01N.20/JK*01 W.06,JK*01W.04/JK* 01,JK*01N.20/JK*01,JK*01W.04/JK*01W.06,*Jk(a+b+)at *JK*01/JK* 02,*Jk(a+b-)at *JK*01/J K*01,*Jk(a+^{W}b+) at *JK*01N.20/JK*02,JK*01W.06/JK*02,*Jk(a-b+) at *J K*02/JK*02* and Jk (a+^{W}b) at *JK*01W.01/JK*01W.06* respectively.

Duffy (FY) Prevalence: The molecular characterization of Duffy phenotypes in the blood donor population of Kenya has signified that there are three FY phenotypes and variants(allelels) associated with this system. .They include the FY*Null GATA regulatory box variant *FY*02N.01/FY*02N.01* or Fy(a–b–); this was found in found in 101/108 (93.52%), followed by a heterozygous type *FY*01/FY*02* and FY c.–67T>C that predicted Fy(a+b–) or Fy(a–b+) at 3.70% (4/108) while *FY*02/FY*02N.01* predicted Fy(a–b+) at 2.78% (3/108). The Null phenotype is associated with complete deletion of the FY gene. These antigens of this system are of medical importance because they are associated with alloimmunisation when introduced to those who lack them. Looking at the current data of this study, there were other phenotypes of this system that were of low frequency Fy (a–b+) 3.70% and Fy (a–b+) 2.78% that were revealed. However, because they are of low prevalence, it does not mean they cannot cause alloimmunisation or antibody production which is the cause of haemolytic transfusion reactions and HDFN. The genotyping or sequencing of this Blood Group System has revealed the molecular basis of Duffy phenotypes in Kenya and thus provided scientific evidence that will inform on the testing protocol for the identification of these variants in the donor population such as extended phenotyping and clinical management of those who require continuous transfusions.

Prevalence of Dantu Variants: Dantu Blood Group System is associated with three types of variants: It yet to be established which one(s) of the three variants is in Kenya. Therefore, due to the limitation of knowing which Dantu variants are frequent in Kenya, this study was limited in the molecular characterization of Dantu variants due to lack of a reference gene to align them. However, this study drawback in the molecular characterisation and identification of Dantu variants does not limit more research in the field to determine the prevalence and provide more data regarding this phenotype. Considering that it is a rare variant in the world population, documentation of its prevalence is essential as well as learning more of associated clinical significance in depth.

Novel variants: This study revealed ten novel variants confirmed by ID numbers and copy variant (Amino Acid) change (KLF1,CD55,C4A;C4B;C4B_2; C4B;C4B_2;SLC35C1, KEL, SLC4A1, ACHE, ABCG2, CR1, and SLC29A1). Some of the Novel variants revealed are of clinical importance such as Kell, Augustine and JR are of clinical significance in transfusion and pregnancy. This is because they have associated in causing haemolytic transfusion reactions and HDFN. Anti Kell and Anti-AUG2 have both been linked to causing haemolytic transfusion reactions and severe haemolytic disease of the foetus and new-born The AUG phenotypes need to be identified both in donors and patients to reduce alloimmunisation. There is need to have robust documented procedures and guidelines entailing how these phenotypes will be identified

5.3 Conclusion

This study outcome is the first one of this kind to be documented in Kenya. Basing on the study findings; molecular characterization of red cell variants. The results of this research has confirmed presence of red cell variants and phenotypes associated with the selected blood group systems. The study has further revealed the existing sub types and weak forms associated with these blood group systems thus confirming their molecular basis. The blood group genotyping carried out during this research has gone ahead to show the prevalence of the predicated phenotypes, subtypes and weak red cell variant for randomly selected Kenyan blood donors; it has also revealed the mutation location and the alleles responsible. Most of the mutations are associated with single nucleotide variation, deletion, and insertion. The study has also revealed that red cell variants in the selected voluntary blood donors in Kenya show variation in comparison to other population and that there are novel variants that are unique (non-synonymous) since they are not yet to be documented in other populations The findings indicates a high possibility of red cell antigens clinical alloimmunization for recipients of transfusion and transplantation and RhD negative women of child bearing age. The outcome is useful in the investigation of red cell antigens alloimmunization especially in ongoing and

massively transfused patients,(Sickle cells, cancer cases among others), RhD- women of child bearing age and antigen/variant matching as well as development of a reference gene bank in Kenya.

In the ABO system: O was the most genotyped at 52% genotyped, B at 14 % , A1 at 12% , A2 at 5.6%, Ax at 4.6% , B/B3 at 4.6%, weak A2 at 2.3% and <1% were typed as A1B and A2B. For the RHD gene; 30% genotyped as D+, 26% as D-, 10.2% as weak D, 4.6% as Del, with a number of other weak variants reported including 1% DAU6.

RHCE System: For the RHCE gene, 28% genotyped as c+e+,17.6%, as C+V+VS+c+e+hrB+, 14% as C+c+e+,13% as V+VS+c+e+hrB+,11.1% as C+V+VS+c+hrB+, 6.5% as JAL+Partial _c/VS+^{W}hrB+$^{W/-}$hrS+$^{W/-}$For the MNS system: 40% genotyped as N+s+, 36.1%

MNS System: M+M^{c}+N+ s+, 21.3% as M+s+ and 2.8% as M+M^{c}+N+S+s+.

Kell System: For the KEL system: 76.9% genotyped as k+, 15.7% as k (KEL2) Jsa (KEL6) Jsb (KEL7), and 22% as K + k+.

Kidd System: Kidd genotyping predicted 28% Jk(a+b-), 24.1% Jk(a+b+), 22.2% Jk(a+b-), 17.6% as Jk(a+^{W}b+), 6.5% as Jk(a-b+), with 1.9% as Jk(a+^{W}b-).

Duffy System: For the Duffy system, the FY null Fy (a-b-) genotype was the most prevalent with ~90% of samples observed with this genotype.

Dantu System: There was limitation in the characterization of Dantu variants because there are three different variants that are associated with Dantu and is not yet determined which one is in Kenya; thus, this will require more data and research to establish a reference gene to align these variants.

Novel Variants: A number of novel non-synonymous variants were also identified in the generated data which includes blood group phenotypes that are associated with potential immunologic significance in transfusion and obstetrics such as KEL and Augustine Blood Group Systems .This study has also generated a distribution of red cell variants for randomly selected Kenyan donors in comparison to other datasets plots among the nations which has

shown that there is limited data of blood group phenotypes and genotypes in Sub –Saharan Africa.

5.4: Recommendation

This study is the first of this kind to be conducted and documented in Kenya. The generated data is very crucial in evidence decision making by the policy makers (Ministry of Health, service users (Blood Transfusion and Transplantation Service, blood banks and clinicians), beneficiaries (patients), Blood donors and stakeholders/partners (funding agencies, learning institutions and researchers). If the generated evidence is implanted, it will also be able to identify gaps that can inform areas of further research and more attention is required

5.4.1: Study Findings to Service Users and Beneficiaries:

The service users are a mixture of experts to include those working at the blood transfusion service, blood banks, transfusing hospitals (private, faith based and public), learning institutions and recipients (patients and students.

National blood service and blood banks: The generated blood group genotyping the data will form a reference in guiding the choice blood groups phenotyping methods, generating a serology red cell phenotyping panels and initiating an extended phenotyping in donors to ensure that no phenotypes including the weak ones are missed in donations; reviewing of developing new testing guidelines and implementing them to the latter. It also useful evidence to initiate a blood group reference laboratory that will be useful in supporting the investigations of transfusions complicated cases

Donors and recipients(patients): The generated data also forms evidence which can be useful in guiding in the antigens identification and matching of donor and recipient blood in the provision of matched units of blood which is safer especially in those who receive ongoing transfusions (SCD, obstetrics and oncology). For the clinicians, the revealed blood group phenotypes will assist in the appropriate use of blood considering the sub types and weak variants beyond the common ABO, D antigens that are of clinical significance in transfusion

in terms of alloimmunisation. This data will also assist in implementing the blood management guidelines as well as reviewing or developing new ones.

Higher Learning Institutions: In the higher learning institutions the research findings will form the basis of paradigm shift in the training using this data for Kenyan reference. For many decades the training on blood transfusion in the middle level medical technical and higher learning instructions has been referring to other studies that was carried in other nations.

Stakeholders/partners/researchers: This study is outcome is very important to stakeholder and researchers in terms of funding for further research and implementation and monitoring and evaluations. It will also form the basis for advocacy for more funding in terms of change of practice in terms of embracing new testing protocols and clinical practice. The evidence can be used by researchers to write funding proposals for additional research in this field.

Summary Recommendation:

The outcome of this study recommends the following:

1. Generation of red cell genomic library for Kenya
2. More research on this field and its extension to include neutrophils and platelets that are also implicated in alloimmunization and to augment the gene data base support
3. Initiation of a molecular blood grouping reference Laboratory in Kenya and East Africa.
4. The study recommends funding allocation in this field of science to support more research in the country in regard to blood groups genetic and precision medicine
5. More research work in Blood group genetic vulnerability in regard to infectious diseases.
6. Introduction of extended Serological phenotyping for alloimmunized patients in the clinical settings-blood banks/transfusing facilities
7. Directed screening of donors for antigen-negative rare blood units for patients should carried out to minimize the danger of reacted adverse transfusion haemolytic and organ rejection reactions and HDFN.

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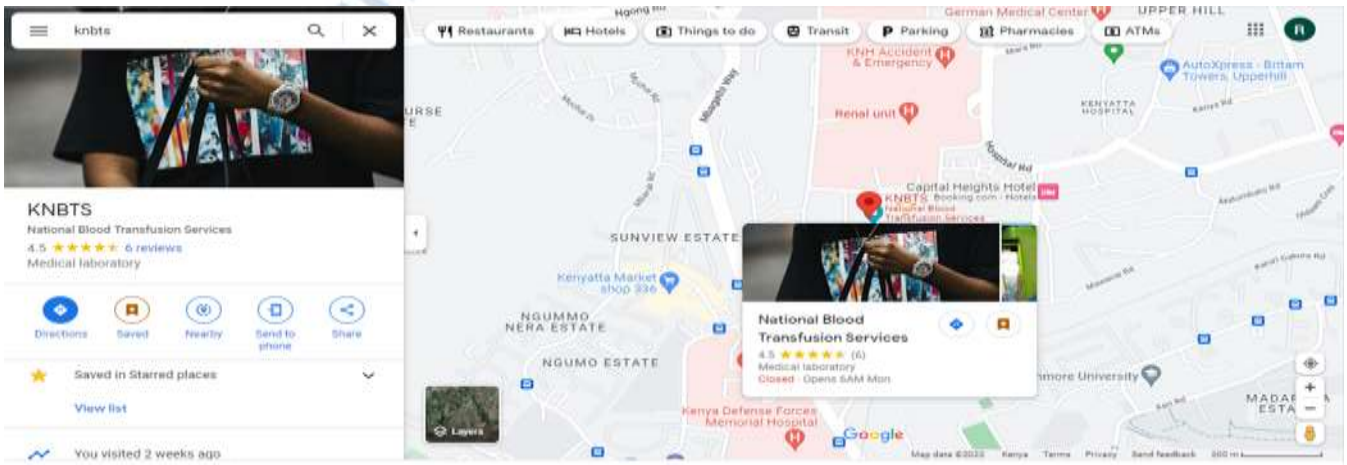
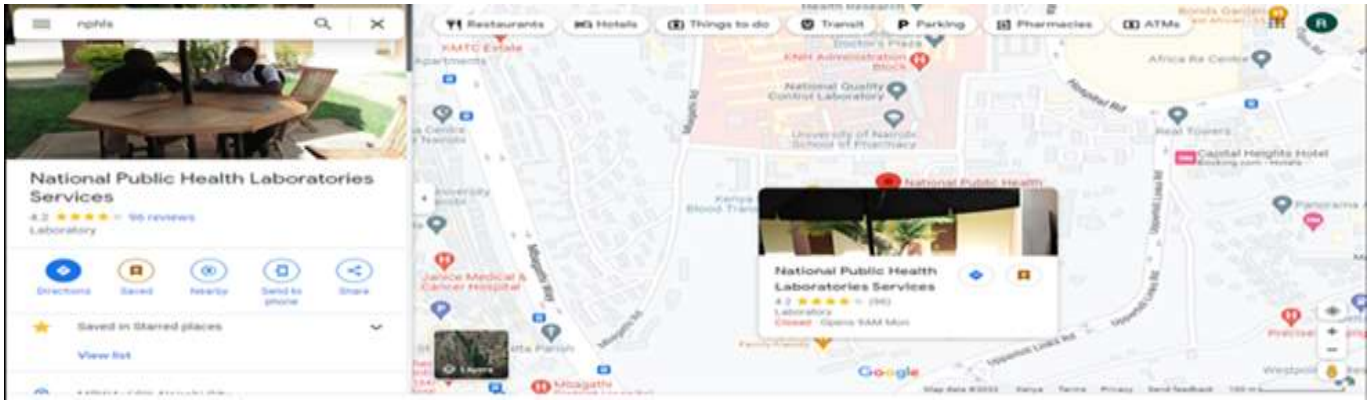
APPENDICES

Appendix I: Kenya National Blood Transfusion Testing Laboratory Location



Map Link to Kenya National Blood Transfusion Services/Tissue and Transplant Authority



Link to National Public Health Laboratories Services

Link to Australia Red Cross Lifeblood Brisbane

Appendix II: Neo ABO/Rh Blood grouping Automated Procedure

ABO/Rh Grouping Using Neo Immucor Automated Blood Grouping Machine

Automated serology blood grouping is a procedure of determining the presence of blood group antigens using an automated platform and commercially acquired antisera

1. **Principle:** Solid phase technology is based on the principle of solid-phase red cell adherence (SPRCA). SPRCA is a method used to detect antibodies by employing either an indirect or direct antiglobulin procedure. In indirect antiglobulin procedures, known antigens are bound to the surface of polystyrene (plastic) microtitre wells. Once the antigens are bound to the surface of the microtiter wells, the microtitre wells can be used to capture specific antibodies

from the samples being tested. After a brief washing step, an anti-immunoglobulin coated Indicator Red Cell is employed to demonstrate the presence of the antibody.

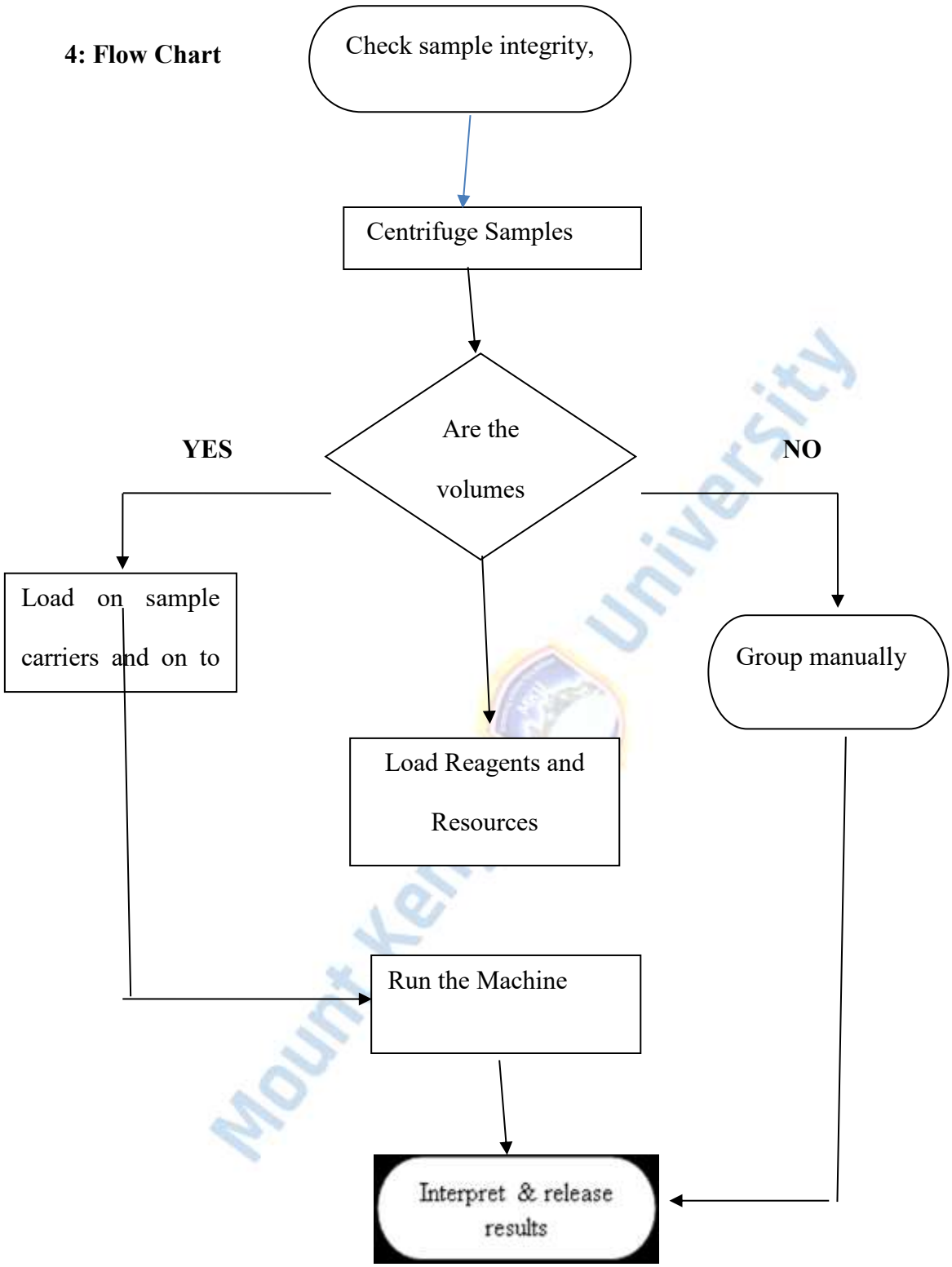
2. Materials and Reagents and Equipment:

1. Bar coded Round-bottomed microplates
2. Labels,
3. Barcode scanner
4. Barcode printer
5. Neo sample carrier
6. Sample diluent
7. Diluent container
8. System concentrate
9. Immunoclone Anti A IgM, Anti B IgM, Anti AB IgM
10. Anti-D Novaclone
11. Immunoclone Anti D Rapid
12. Immunoclone Rh Hr control
13. Capture Indicator Red cells
14. Reference cells kit- $A_{(1)}$,B
15. Capture Liss
16. corQC Extended Standard cells
17. Capture-R-Select plates
18. Gloves. 19:printer

3: Safety Precautions

- Treat all human material as potentially infectious.
- Use appropriate protective clothing when handling samples i.e. gloves, lab coats.
- Any spillage of human material should be handled according to the organization's procedure on handling bio-hazardous spills.
- General laboratory safety guidelines must be observed at all times.

4: Flow Chart



4.1.1: Sample preparation and requirements

i. Ensure that the Donor Samples are in EDTA tube, coded and 4 ml in volume (Do not use clotted or haemolysed samples)

ii. Inspect samples for haemolysis, clots and volume. Set aside the unacceptable ones, if possible, group them manually.

iii. Centrifuge samples at approximately 4000 rpm for 8 minutes

4.1.2: Reagent preparation and requirements

i. Refer to the reagents package insert for storage and other requirements

ii. Remove reagents from the fridge if required

iii. Inspect the reagents for expiration dates, appearance and volume, and replace if necessary

iv. Insert a magnetic bead into each new bottle of cells before loading.

v. Verify the QC for reagents for the day has been performed and results are satisfactory. Refer to the online equipment user instructions (Daily maintenance of instrument)

4.1.3: Loading of Resources and samples

i. Numerically, arrange the samples in sample racks according to donation numbers. Start with the sample having the lowest donation number in each team by putting the first sample in position A of the rack and the last in Position O of the same rack

ii. Place all the reagents on to the reagent rack ensuring that the “longer” barcode label is clearly visible on the view window

iii. Initiate the “start routine” by clicking on the start assistant (“the running man”)

iv. Load reagents on bay 14. The barcodes will be scanned by the instrument

v. Uncap the samples and ensure that the barcodes are clearly visible on the view window of each rack

vi. Load the sample racks containing the samples from sample bay 2 onwards. The barcodes will be scanned by the instrument. In the event that a sample is not automatically scanned, scan it manually using the scanner. Alternatively, order the tests manually by clicking on the selected assay, and then CLICK ALL…

vii. Click the icon of "running man" on the task bar then select Load resources. Press on resource name and check on the missing resources (marked with red!) and load using the appropriate button above red!

viii. Load plates via plate loading tower. Barcodes will be scanned by the instrument.

ix. Press start button for the running to commence. Once testing has been completed, the bays with sample racks that do not contain any samples requiring weak d testing will illuminate green, while those with samples for weak d testing will illuminate amber.

4.1.4: **Loading of capture plate for weak D testing**

i. Confirm the list of samples that are to be tested for weak D

ii. Select capture assay from resource overview, press plates, mark appropriate assay name, press strip selection and take note of the number of strips needed.

iii. Fix the required number of strips on strip/ plate holder

iv. Deselect unused strips on capture plates from step 3 above and click done

v. Click on all plates (highlighted in blue) in Resource Overview to start. Press start.

4.1.5: Review and printing of Results

i. Look for all the completed results under

ii. Review the images (agglutination strength) for result interpretation as shown in step 11.8 and approve. Edit results with an agglutination strength of above 60%

iii. Print results by highlighting all results that need to be printed, select list then click on report, and then select print.

iv. Review all results on the print out and record in the lab register, FRM LAB 002.

4.1.6: Interpretation of Results

i. Agglutination indicates the positivity or presence of the antigens for the corresponding antibody.

ii. The absence of agglutination indicates the negativity or absence of the antigen and appears as homogeneous mixture in the tube.

iii. Grading of Agglutination Reactions

Grading of Agglutination reactions		
Grade	**Significance**	**Interpretation**
4+	1 large clog	**Positive**
3+	2 or 3 clogs	
2+	Several minor clogs with clear supernatant	
1+	Several minor clogs with unclear supernatant	
w	Coarse suspension	
H	Incomplete/complete hemolysis(Positive reaction)	
Zero or -	Smooth suspension (Negative reaction)	**Negative**

N/B: Always compare Results for ABO Reverse grouping with those for ABO Forward blood grouping

If any discrepancy is found, refer to the procedure for resolution of ABO discrepancies

Appendix III: Pharmacy and Poisons Board Export Permit Approval

REPUBLIC OF KENYA PHARMACY AND POISONS BOARD

EXPORT PERMIT

Document	321J - EXPORT PERMIT
Document Type	2 - Permit
Application Reference No : 2021CPPB321J0000292458	Version No : 1
Master Approval No	
Master Approval Version No	
UCR Number	UCR2100020938

Application Status

Approval Status :Approved-Pending Release Application Date :2021-11-23 14:49:25.385 Amended Date :
Expiry Date :2022-11-29 13:07:39.596 Approval Date :29/11/2021 13:05:53

Applicant Details

Name :KENYA MEDICAL RESEARCH INSTITUTE

PIN :P051092793H	Application Code :KMR
Address :54840 00200	Country :KENYA
Contact Person :NA NA	Email : ezekiel.muinde@yahoo.com

Consignee Details

Name :QUEENSLAND UNIVERSITY OF TECHNOLOGY

PIN :P000000000N	OGA Ref No :
Physical Address :Queensland	Physical Country :AUSTRALIA
Postal Address :	Postal Country :
Telephone :54647444848	Fax :54647444848
Email :rFlower@redcrossblood.org.au	Sector of Activity :
Warehouse Code :	Warehouse Location :

Importer Details

Name :QUEENSLAND UNIVERSITY OF TECHNOLOGY

PIN :P000000000N	OGA Ref No :
Physical Address :Queensland	Physical Country :AUSTRALIA
Postal Address :	Postal Country :
Telephone :54647444848	Fax :54647444848
Email :rFlower@redcrossblood.org.au	Sector of Activity :
Warehouse Code :	Warehouse Location :

Appendix IV: Objectives Analysis

Objective Analysis							
	Objective	**Variable**	**Indicators**	**Measurement**	**Measurement scale**	**Method of Data collection**	**Method of analyzing Data**
1	To determine ABO genotype distribution in voluntary blood donors of Kenya applying molecular typing	Dependent	Red cell Antigens and variants	Antigens and variants presence	Ordinal	Laboratory and sequencing	Stata version 16 Descriptive statistics Workbench software version 8.5 (QIAGEN prescriptive statistics
2	To determine weak D variants in voluntary blood donors of Kenya applying molecular typing	Dependent	Red cell Antigens and variants	Antigens and variants presence	Ordinal	Laboratory and sequencing	Stata version 16 Descriptive statistics Workbench software version 8.5 (QIAGEN prescriptive statistics
3	To establish the frequency of rare red blood cells variants (c,e, MNS, Kell, Kidd and Duffy) in voluntary blood donors of Kenya using molecular typing	Dependent	Red cell variants	variants presence	Ordinal	Laboratory and sequencing	Workbench software version 8.5 (QIAGEN prescriptive statistics
4	To determine the prevalence of Dantu variants in voluntary blood donors of Kenya using molecular typing	Dependent	Red cell variants	variants presence	Ordinal	Laboratory and sequencing	Workbench software version 8.5 (QIAGEN prescriptive statistics

Appendix V: Material Transfer Agreement

Material Transfer Agreement

Australian Red Cross Blood Service

And

Kenya National Blood Transfusion Service

This Agreement is made on the day of 2018

Parties

The **Australian Red Cross Society,** a body corporate established by Royal Charter on June 28, 1941, directing its operations through the **Australian Red Cross Blood Service** (ABN 50 169 561 394) and having an address at 3/417 St Kilda Rd, Melbourne Victoria, 3004 (**Blood Service**) **and**

Kenya National Blood Transfusion Service (**KNBTS**), a Division in the Ministry of Health of Health Act 2017

Background

A The Blood Service, or KNBTS as the case may be (**providing Party**), would like to provide the other party (**Recipient**) with the Material specified at **Item 4** of **Schedule 1** (the **Material**).

B The Parties have agreed to enter into this Agreement for the provision of the Material to each other.

The parties agree that:

1 Term

Start Date

1.1 This Agreement begins on the date specified in **Item 2** of **Schedule 1** (**Start Date**).

End Date

1.2 Unless terminated otherwise in accordance with, this Agreement expires on the End Date set forth in Item 3 of Schedule 1. **Clause 8.**

Extension

1.3 The Recipient may elect and agree to extend the term of this Agreement for any set period beyond the End Date. Any extension of the term is subject to the prior approval of the Providing Party and such approval is at the exclusive discretion of the Providing Party.

1.4 In the event that a party wishes to extend the term, that party will notify the other party, in writing, of its intention one month prior to the End Date.

2 Relationship

Independent contractor

Except for the relationship between the contracting parties, this Agreement does not create any partnership, employment, agency, or fiduciary relationship.

Effect of provision of Material

1.3 The Parties acknowledge that this Agreement:

1.3.1 in no way contemplates, intends or effects the sale by the Providing Party, or the purchase by the Recipient or any other person, of such Material; and

1.3.2 Does not operate so as to transfer or pass rights in such Material to the Recipient other than the rights to perform the Purpose as detailed within Schedule 1.

3 Provision of Material

Provision of Material

3.1 Subject to **clauses 3.4, 3.5** and **3.6**, the Providing Party will provide the Material over the term of this Agreement.

3.2 The Recipient shall initiate the transfer of the Material by submitting the Order Form to the Providing Party.

3.3 The providing Party will transfer to the Recipient the quantity of Material as detailed within the Order Form submitted by the Recipient, provided that such quantity of Material is at no time greater than the Quantity specified in **Item 5** of **Schedule 1**.

3.4 Notwithstanding any request for Material pursuant to **clause 3.3**, the Parties acknowledge that the providing Party's obligation to transfer the Material to the Recipient under this Agreement is at all times subject to the availability of the Material and is at all times at the sole and absolute discretion of the Providing Party.

3.5 The parties acknowledge that the Material as provided by the Blood Service is not supplied as an *in vitro* diagnostic medical device (**IVD**), within the meaning of the *Therapeutic Goods (Medical Devices) Regulations 2002* (as amended or replaced from time to time).

3.6 If insufficient or no Material is available for transfer to the Recipient at the time the Order Form is submitted by the Recipient, the providing Party will inform the Recipient to determine whether the order and/or delivery time can be revised to satisfactorily meet or partially meet the request on the Order Form or whether the order must be cancelled.

3.7 For avoidance of doubt, the providing Party is not liable to the Recipient for any failure to source or provide or any delay in providing the Material.

Due skill, care and diligence

3.7 The Party Giving the Fabric will do so with all due aptitude, care, and tirelessness and in agreement with by and large acknowledged commerce and proficient hones...

Obligations of the Recipient

Collection of the Material

3.8 The providing Party agrees to transfer the Material to the Recipient from the Providing Party's premises specified in **Item 6** of **Schedule 1** (**Providing Party Premises**) at the times agreed by the parties.

3.9 The Recipient agrees that risk in the Material will pass to the Recipient upon collection by the Recipient or its contracted carrier for the transport of the Material. Any and all responsibility and liability of the Providing Party for handling and storage of the Material shall cease upon collection of the Material by the Recipient or its contracted carrier from the Providing Party Premises.

3.10 The Recipient must insure the Material for loss of, or damage to, the Material in favor of the Recipient.

3.11 The Recipient indemnifies the Providing Party from and against any and all obligation, misfortune, hurt, harm, taken a toll or cost (counting legitimate expenses on a full repayment premise) however arising, that the Providing Party may suffer, incur or sustain, once the risk in the Material passes to the Recipient.

Use of the Material

3.12 The Beneficiary must utilize the Fabric exclusively and as it were for the Reason indicated in Thing 1 of Plan 1 and must not utilize the Fabric for any other reason, counting any commercial reason.

3.13 Where the use of the Material provided for the Purpose is intended for the manufacture of an IVD, the Recipient will be responsible for compliance with all regulatory requirements in respect of IVDs.

3.14 The Recipient must comply with all applicable laws, regulations and guidelines regarding the use of the Material, including (where relevant) those concerned with importation of the Material.

3.15 Where any morals endorsement of any individual or body is required to utilize the Fabric, the Beneficiary must:

3.15.1 obtain the relevant ethics approval; and

3.15.2 Comply with all conditions of that ethics approval.

3.16 The Beneficiary must comply with any composed headings from the Giving Party in connection to utilize of the Fabric, counting but not constrained to the pulverization or transfer of the Fabric.

3.17 The Beneficiary must not investigate, dissect, adjust, conduct tests on the Fabric or utilize the Fabric on human subjects, unless allowed by the Reason, and in such case, as it were to the degree allowed by the Reason.

Possession and storage

3.18 The Recipient warrants to the Providing Party that the Recipient has all regulatory approvals, licenses or consents required by law, government agencies or other bodies, relating to the possession of the Material and the use of the Material for the Purpose.

3.19 The Recipient must ensure that the Material is stored in accordance with all applicable laws, regulations and guidelines and is only accessible by persons authorized by the Recipient to have access.

3.20 The Recipient must not provide the Material or any part thereof to any third party without prior written consent of the Providing Party, and such consent is at the exclusive and absolute discretion of the Providing Party.

3.21 Where consent is provided by the Providing Party, the Recipient must ensure that the Material (or part thereof) is provided to the third party subject to the condition that the Material (or part thereof) must not be provided to any other person. Notwithstanding such consent, the provision of Material to a third party is at the risk of the Recipient

and the indemnity in **clause 7** will apply in the event of any loss, damage, action, proceeding or demand arising from the provision to, or use by, a third party of such Material.

Safety

3.22 The Beneficiary acknowledges any and all chance and obligation related with the Fabric, upon receipt of the Material.

3.23 The Beneficiary recognizes and concurs that the Fabric may be poisonous, may contain irresistible specialists, or other substances that are perilous or unsafe, or hurtful to people or property.

3.24 The Beneficiary warrants to the Giving Party that at all times it'll be dependable for the secure utilize, dealing with, capacity and transfer of the Fabric, in such a way to guarantee that the Fabric will not cause any hurt, harm or misfortune to any individual or to any property.

3.25 The Recipient warrants to the Providing Party that the Recipient will use, handle, store and dispose of the Material with caution, all due skill, care and diligence, and in accordance with accepted professional, business practices and all applicable legislation and/or regulations in any experimental work, since not all characteristics of the Material are necessarily known.

3.26 The Recipient warrants to the Providing Party that, given the nature and characteristics of the Material, the Recipient:

3.26.1 is aware of all matters concerning the safe handling and storage of the Material; and

3.26.2 Has all facilities and processes required for the safe use, handling storage and disposal of the Material.

3.27 Any part of the Material that is not used by the Recipient, or that must be disposed of in accordance with any clause under this Agreement, must be destroyed by the Recipient in the manner required by all applicable laws or regulatory agencies for the disposal of potentially bio-hazardous waste.

4 Confidentiality and privacy

General obligation

4.1 The Beneficiary must not uncover Private Data, but on the off chance that the revelation is required by law.

4.2 Nothing in this Understanding confers upon the Beneficiary any right or permit to any portion of the Fabric or Private Data.

Compulsion to disclose confidential information

4.3 If the Beneficiary gets to be mindful of any steps taken, being taken, or considered, to compel the Beneficiary or its specialists or representatives to reveal Private Data of the Giving Party, the Beneficiary must:

4.3.1 to the degree allowed by law, concede and constrain the revelation with a see to protecting the secrecy of the Private Data as much as possible;

4.3.2 promptly inform the Giving Party; and

4.3.3 Do anything sensibly required by the Giving Party counting the institution and conduct of lawful procedures at the Giving Party's course and cost to restrict or confine that disclosure.

4.4 Except with the prior written consent of the Providing Party, the Recipient must:

4.4.1 keep the Secret Data mystery and confidential;

4.4.2 not uncover to any individual or make known in any way any of the Secret Information; and

4.4.3 Not apply for any patent or other statutory protection of the Confidential Information.

4.5 The Recipient's obligations in respect of Confidential Information in **clauses 5.3** and **5.4** shall not apply in respect to information which:

4.5.1 is now or later becomes publicly available through no fault of the Recipient;

4.5.2 the Recipient obtains from a third party not under an obligation to the Providing Party with respect to such Confidential Information;

4.5.3 the Recipient already has in their possession as shown in their written records prior to the date of disclosure under this Agreement; or

4.5.4 is required by any law, rule, regulation, order, decree or subpoena or other judicial, administrative or legal process to be disclosed, provided that, where practicable, Recipient promptly notifies the Providing Party of such legal requirement to disclose the Confidential Information so that the Providing Party may take appropriate steps to protect the confidentiality of such information.

Compliance with privacy laws

4.6 The Recipient must comply with:

4.6.1 all relevant privacy, health records or similar legislation and regulations which the Providing Party is required to comply with; and

4.6.2 any kind of requests which the Giving Party may make in composing to the Beneficiary, which may be sensibly required to guarantee the Giving Party complies with its commitments emerging beneath any pertinent security, wellbeing records or comparable enactment or controls.

Continuing effect

4.7 This **clause 5** will continue to have effect after the expiry or termination of this Agreement.

5 Publications

5.1 The Recipient may not make Publications containing or referring to the use of the Material for the Purpose, without the prior written consent of the Providing Party.

5.2 Where approval by the Providing Party is given under 6.1 and the Publication does contain, or where the Recipient cannot guarantee that the Publication does not disclose Confidential Information of the Providing Party, the Recipient must provide a copy of the Publication to the Providing Party at least 30 days before forwarding or presenting to any person not bound by the confidentiality obligations set out in **clause 5**.

5.3 The Providing Party will at all times have absolute discretion to determine whether the Publication contains Confidential Information or whether the Recipient must comply with **clause 6.2**.

5.4 Acting reasonably, the Providing Party may, within that 30 day period require the Recipient to remove specified Confidential Information from the Publication.

5.5 If the Beneficiary has not gotten any comments from the Giving Party on the proposed Distribution inside 30 days of giving take note to Giving Party beneath clause 6.2, the Beneficiary may make the Distribution.

5.6 The Recipient must acknowledge the role of the Providing Party in any Publication containing or referring to the Materials.

6. Indemnity and Warranty

6.1 Unless something else indicated in this Assentation, not one or the other the Giving Party nor any of its workers, associates, operators, providers, third-party data suppliers, shippers, licensors, or other agents (each a "Giving Party" and collectively, the "Giving Parties") give any guarantees of any kind, whether communicated or suggested, statutory or something else, relating in any way to the subject matter of this Understanding. These guarantees incorporate, but are not restricted to, guarantees of merchantability, wellness of them.

6.2 Any term that's consolidated into this Understanding by a statute which statute maintains a strategic distance from or forbids contract arrangements barring or adjusting the application of or obligation beneath such a term will be considered to be consolidated into this Understanding. Be that as it may, the Giving Party may select to restrain its risk for any breach of the term to the resupply of the Fabric or installment of the taken a toll of having the Fabric provided once more on the off chance that that statute grants it.

6.3 The Beneficiary is mindful for and must repay the Giving Party and its officers, representatives, and specialists against any obligation, misfortune, harm, or fetched (counting legitimate expenses on a full repayment premise) resulting from any of the following:

6.3.1 the Recipient’s use of the Material;

6.3.2 any use of the Material by a third party that receives the Material through the Recipient;

6.3.3 any negligence or other wrongful act or omission of the Recipient or the Recipient's staff, employees, or agents or of any other person for whose acts or omissions the Recipient is vicariously liable;

6.3.4 any damage to people, counting damage coming about in passing and financial misfortune caused by the acts or exclusions of the Beneficiary or the Recipient's staff, representatives, or specialists or of any other individual for whose acts or exclusions the Beneficiary is vicariously at risk; and/or

6.3.5 Any breach of this Agreement by the Recipient.

6.4 The degree to which any activity, continuing, claim, or request emerges out of the carelessness or other wrongful act or exclusion of the Giving Party or any individual for whose acts or exclusions the Giving Party is obligated decreases the Recipient's obligation beneath this clause.

6.5 This clause's indemnification continues even after this Agreement is terminated or expires.

6.6 A Recipient's total liability to the other party under this **clause 7** will be limited in the aggregate and total to an amount of (AUD) one million dollars (AUD $1,000,000).

7 Termination or Expiry

7.1 This agreement can be terminated on mutual agreement in a written or as per clause 8.

7.2 Notwithstanding anything in this Agreement, the Providing Party may terminate this Agreement immediately if:

7.2.1 the Recipient uses the Material for any purpose which is not the approved Purpose;

7.2.2 the Recipient's use of the Material is outside the scope of the Purpose as contemplated by this Agreement;

7.2.3 a warranty by the Recipient is inaccurate; or

7.2.4 The Providing Party ceases, or indicates that it is about to cease, providing the Material for any reason.

7.3 The Providing Party must give the Recipient a written notice which specifies the event causing the immediate termination.

7.4 Either party may terminate this Agreement by giving the other party written notice at least one month in advance.

On termination or expiry

7.5 On termination or expiry of the Agreement the Recipient must, unless as otherwise directed by the Providing Party:

7.5.1 pay to the Providing Party within 14 Business Days of the date of termination or expiry, any outstanding monies;

7.5.2 immediately destroy that part of the Material in the Recipient's possession, in accordance with **clause 4.19**;

7.5.3 use its best endeavors to immediately retrieve any Material in the possession of a third party or where retrieval is impractical ensure that any Material in the possession of a third party is immediately destroyed by that third party in accordance with **clause 4.19**;

7.5.4 For the avoidance of doubt, the Recipient must not store the Material beyond the End Date without the express written consent of the Providing Party.

7.6 Clause 8.5 of this agreement shall be binding even after this agreement time frame has laps.

8. Notices

Address and fax number

8.1 Notice shall be given to any party of this agreement if:

8.1.1 delivered or posted to that party at the address stated in **Item 7** of **Schedule 1**; or

8.1.2 Faxed to that party at the fax number stated in **Item 8** of **Schedule 1**.

8.2 A party may inform the other party in composing three trade days some time recently making any changes to its address recorded in Thing 7 of Plan 1 or fax number recorded in Thing 8 of plan.

Miscellaneous

Assignment

8.3 A Party may as it were allot or novate its rights or commitments beneath this Understanding with the express earlier composed assent of the other Party.

Variation

8.4 This Assentation may as it were be shifted by assentation in composing of all parties or something else as explicitly given in this Assentation.

Severability

8.5 If any clause or part of a clause of this Agreement is or can be read in a way that makes it illegal, unenforceable or invalid, then:

8.5.1 if that clause can be studied in a way that produces it lawful, enforceable and substantial, it must be examined in that way; or

8.5.2 That clause or part which is illegal, unenforceable or invalid is to be treated as evacuated from this Understanding, but the rest of this Assentation isn't influenced.

Entire agreement

This agreement contains the complete assentation of the parties in connection to the Fabric.

Governing law

8.6 This Assentation is administered by the law of the State and nation indicated in Thing 9 of Plan 1. The parties experience the non-exclusive locale of the courts in that location.

8.7 Special conditions

8.8 The special conditions specified in **Item 11** of **Schedule 1** (if any) apply to this Agreement.

9.1 Definitions and Interpretation

Definitions

9.1 In this Agreement:

Business Day means any weekday that is not gazette as a public holiday in the town/city and State specified in **Item 10** of **Schedule 1**;

The terms **"confidential information**" and "donor data," "names," "inventions," "discoveries," "facts," and "ideas," among others, refer to all information pertaining to the Material that is by its very nature confidential. chemical composition or detailing, methods, items, models, forms, know-how, schedules, determinations, drawings, exchange insider facts, innovation strategies, computer programs, works for which copyright exists, and data (at whatever point it was gotten) in connection to the Giving Party's commerce, operations, techniques, property, or clients that's:

(a) confidential in fact;

(b) reasonably regarded by the Providing Party as confidential; or

(c) Specified as being confidential in a written notice from the Providing Party to the Recipient.

If the follow happens then the information is not confidential:

(a) unless a breach of confidentiality under this Agreement brought it into the public domain, it is or becomes part of the public domain;

(b) it comes from a third party legally and without violating confidentiality;

(c) the Recipient's written record indicates that it knew about it prior to the disclosure date;

(d) it was developed independently by a recipient employee who was unaware of the disclosure required by this agreement;

(e) if required to be disclosed by a court, rule, government policy, or stock exchange policy, provided that the party disclosing the information promptly notifies the other party of the requirement; or (f) it must be disclosed in accordance with this Agreement.

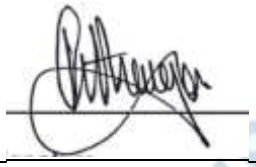
Notice incorporates any report or correspondence required, allowed or alluded to in this Understanding,

Order Form means the order form, as amended from time to time, issued by the Providing Party and completed by the Recipient for the purpose of specifying the Material and the quantity of Material required by the Recipient,

Publication implies any frame of communication of data, counting but not constrained to any report, theoretical, paper, article, original copy, blurb, web posting, introduction slides, unique, video, diagram, instruction fabric or other revelation, in printed, electronic, verbal or other frame, and

Purpose means use of the Material for the purpose specified in **Item 1** of **Schedule 1** and not for any other purpose, including any commercial purpose.

Execution

Signed on behalf of **Australian Red Cross Blood Service** by its duly authorised representative: **Prof Robert Flower** _____ Signature  _____ Name of Authorised Representative _____ Signature of Witness _____ Date	**Signed** on behalf of the **Kenya National Blood Transfusion Service** by its duly authorised representative:  _____ Signature **Dr. Josephine Githaiga** Name of Authorised Representative  Signature of Witness _____ Date

Schedule 1: Reference Schedule

Item 1	Purpose (Background B and clause 4.5)	1. To perform genetic analysis of received samples for the purpose of conducting a collaborative research project "Molecular typing of blood group variants". 2. To enable the Receiving Party to maintain a DNA library of samples derived from Kenya blood donors to be used for the Receiving Party's own internal research purposes.
Item 2	Start Date (clause 1.1)	On the date this Agreement is fully executed
Item 3	End Date (clause 1.2)	Four years from the Start Date
Item 4	Material (Background A)	Material of the Blood Service to be transferred: Fully sequenced samples Material of KNBTS to be transferred: DNA samples from Kenyan blood donors.
Item 5	Quantity (clause 3.3)	Material of the Blood Service to be transferred: fully sequenced DNA samples – up to 20 per annum Material of KNBTS to be transferred: up to 200 DNA samples from samples with complex or variant serology, per annum

Item 6	Receiving Party premises (clause 4.1)	**Blood Service:** Brisbane Processing Centre 44 Musk Avenue, Kelvin Grove, QLD 4059 **KNBTS:** 29804-00202, KNH Grounds-Hospital Road Nairobi Kenya
Item 7	Addresses (clause 9.1.1)	**Blood Service**: 17 O'Riordan St, Alexandria NSW 2015, AUSTRALIA **KNBTS:** 29804-00202, KNH Grounds-Hospital Road Nairobi Kenya
Item 8	Fax Numbers (clause 9.1.2)	**Blood Service**: (02) 9234 2411 **KNBTS**: (65) 6213 0749
Item 9	Governing Law (clause 10.5)	New South Wales, Australia, if the party raising the dispute for resolution is the Blood Service and Kenya, if the party raising the dispute for resolution is KNBTS.

Item 10	Town and State for the purposes of a Business Day (clause 9.1)	Sydney, New South Wales, Australia where the party having the obligation of an action under the contract is the Blood Service and Kenya where the party having the obligation of an action under the contract is KNBTS.
Item 11	Special Conditions	n/a

Dated 01/11/2018

AUSTRALIAN RED CROSS SOCIETY
(Acting through its Australian Red Cross Blood Service)

And

KENYA NATIONAL BLOOD TRANSFUSION SERVICE

==========

COLLABORATION PROJECT AGREEMENT "MOLECULAR TYPING OF BLOOD GROUP VARIANTS"

This Agreement is made on the 01/11/ 2018

Between:

The Australian Red Cross Society, a body corporate established by Royal Charter dated 28 June 1941, acting through that part of its operations known as the **Australian Red Cross Blood Service** (ABN 50 169 561 394) of 3/417 St Kilda Road Melbourne Victoria 3004 ("**BLOOOD SERVICE**").

Kenya National Blood Transfusion Service, a body established under the Health Act 2017 (Health Act 2017 pg. 272) and having its address at 29804-00202 Nairobi Kenya ("**KNBTS**"); and (each a "**Party"** and collectively "**the Parties**")

Whereas:

The Blood Service wishes to undertake a collaborative project entitled "Molecular typing of blood group variants".

The Kenya National Blood Transfusion Service wishes to ensure the safety of the blood and blood products it provides for transfusion in patients by extended typing of donors.

BLOOOD SERVICE and KNBTS wish to collaborate with each other in the conduct of this project.

It is agreed detailed below

Interpretation

Definitions

"**Background Intellectual Property**" means Intellectual Property owned by the respective Party or Intellectual Property to which the respective Party has rights prior to the Effective Date that is used by the Parties in the course of conducting the Project.

"**Confidential Information**" means all individually identifiable information, personal data and other information identified as confidential by the Parties or which by virtue of its nature ought to be kept confidential, including know-how, methodology, trade secrets, ideas, concepts, technical and operational information, scientific or technical processes or techniques, Project processes, sequences, structure, and organization, customer lists, information relating to the Parties' business, operations, or strategies, intellectual property, information relating to actual or prospective suppliers or competitors.

"**Effective Date**" means the date on which this Agreement is entered into by the Parties.

"**Foreground Intellectual Property**" means Intellectual Property that is invented, created, conceived, developed, generated, first reduced into practice or writing by the Parties or the Parties' employees, personnel, agents or contractors, independently of each other or jointly and/or in collaboration with each other, in the course of or as a result of conducting the Project.

"**Intellectual Property**" means all confidential information, trademarks, benefit marks, exchange names, logos, get-up, licenses, innovations, disclosures, enlisted and unregistered plan rights, copyrights, semi-conductor geography rights, database rights, and all other comparable rights in any portion of the world (counting know-how), counting any enlistment of such rights and applications and rights to apply for such enrolments.

"**Project**" means the programme of work to be conducted for the project as referred to in clause 2.1, as more particularly described in the Project Summary Sheet attached hereto as Schedule 1.

"**Project Period**" means the period commencing on the Effective Date and expiring on 30 June 2021.

"**Samples**" mean complex DNA samples extracted by KNBTS and provided to the BLOOOD SERVICE for the Project

When a word or phrase has a specific meaning, it has the same meaning in any other form; any alterations or re-enactments of statutory provisions are included in references to those provisions;

The Section headings and subheadings in this Agreement are merely for convenience; they are not part of this Agreement and have no bearing on how it should be interpreted.

In this Agreement, unless the context requires otherwise:

The plural or singular number, respectively, is included in words that use either the singular or plural number; All genders are to be referred to as "persons," and businesses and corporations are to be referred to as "corporations."

Any common individual, legitimate substance, affiliation, statutory body, organization, restricted obligation company, joint wander, believe, bequest, unincorporated organization, state, or any political subdivision, instrumentally, organization, or specialist are all alluded to as "persons."; And References to Clauses and the Plan are to the clauses of, and the plans to, this Agreement.

Parties' Responsibilities

Blood Service's Responsibilities

BLOOOD SERVICE will at the request of KNBTS for the purposes of system validation, undertake sequencing as appropriate for determination of blood group variants on DNA samples provided by KNBTS. Testing by BLOOOD SERVICE is subject to provision of extracted DNA from KNBTS and will at all times be subject to BLOOOD SERVICE's discretion.

BLOOOD SERVICE will integrate testing with other activities and with the objective of undertaking testing within four (4) weeks of receipt of a sample. A maximum number of 200 Samples will be tested per annum.

Wherever applicable, KNBTS will be responsible for obtaining the requisite ethics approval for the conduct of the Project from the relevant Committee or Board.

BLOOOD SERVICE acknowledges that KNBTS may itself wish to undertake its own system validation tests on DNA samples for its internal operational purposes, BLOOOD SERVICE agrees at KNBTS's request, to provide to KNBTS similar DNA samples as are available from its database for such use by KNBTS, subject to any requirements of the Blood Service in respect of supply of biological material requiring separate terms relating to the transfer of biological material.

KNBTS's Responsibilities

2.4 KNBTS will at its discretion reasonably endeavour to collect and extract sufficient quantities of the Samples to provide BLOOOD SERVICE for the Project.

Mutual Responsibilities

Each Party will use its reasonable endeavours to undertake their respective responsibilities for the Project in accordance with this Agreement and all applicable laws, regulations, guidelines and conditions imposed by the Institutional Review Board approving the Project.

Each Party will bear its own expenses in undertaking its responsibilities under this Project.

Licence to Use Background Intellectual Property

Each Party agrees to grant the other Party a non-exclusive, non-transferable, perpetual, royalty-free licence to use its Background Intellectual Property for the purposes of conducting the Project and the other Party's own internal, non-commercial research use only.

The ownership of each Party's Background Intellectual Property remains with the respective Party and is not affected by this Agreement. Save as expressly provided in this Agreement in

Clause 3.1, nothing contained in this Agreement shall be implied to grant either Party any right or licence with respect to the other Party's Background Intellectual Property.

Ownership of Foreground Intellectual Property

All Foreground Intellectual Property, results, outcome, data and information invented, created, conceived, developed, generated, first reduced into practice or writing by the Parties or the Parties' employees, whether independently of or jointly or in collaboration with each other, during and/or in the course of conducting the Project or other activities pursuant to this Agreement, shall be owned jointly by the Parties.

In the event that either of the Parties shall consider that any Foreground Intellectual Property generated should be made the subject of any application for the protection of intellectual property rights or commercialization, the Parties shall discuss and agree on the matters relating thereto including but not limited to cost and profit sharing issues.

Neither Party shall file any application for the protection of Foreground Intellectual Property without the prior written consent of the other.

Publication

KNBTS and BLOOOD SERVICE agree that neither Party shall publish any articles or papers in any journals, periodicals or any other media on the Project and disclosing the results and/or outcome of the Project without the participation of the other Party unless the other Party has waived in writing its participation in such publication.

In the event that a Party intends to publish and the other Party has waived its participation in such publication in accordance with Clause 5.1, the other Party may review the draft of the article or paper to be submitted for publication. For the avoidance of doubt, the other Party shall only review the draft for any information that is its Confidential Information, and the publishing Party shall remove such Confidential Information from the draft prior to publishing when informed to do so. If the publishing Party does not hear from the other Party within thirty

(30) days after the publishing Party submits a draft to the other Party for review, the publishing Party shall be free to publish the draft without further reference to the other Party.

Each Party, when publishing any article or paper on the Project, shall ensure that due acknowledgement and credit is given to the other Party and employees of the other Party who had contributed towards the Project.

Each Party agrees that it may be necessary to delay the submission of any articles or papers for publication if such delay is necessary in order to seek protection for the Foreground Intellectual Property, following a decision by the Parties to file for such protection, and in such instances, no submission for publication will take place until such time when the publication will not prejudice the seeking of protection for the Foreground Intellectual Property.

Commercialization

In the event that the Parties are of the view that the results and/or outcome of the Project and/or Foreground Intellectual Property is suitable for commercialisation and exploitation, the Parties shall discuss and agree on how best to commercialise and/or exploit the results and/or outcome of the Project and/or Foreground Intellectual Property.

Neither Party shall commercialise and/or exploit the results and/or outcome of the Project and/or Foreground Intellectual Property without the prior written consent of the other.

For the avoidance of doubt, subject to Clause 5, either Party shall be free to use, without consent from or compensation to the other Party, the results and/or outcome of the Project and/or Foreground Intellectual Property for academic, research and other non-commercial purposes.

Warranties and Representations

Each of the Parties represents and warrants that:

It has full legal capacity and power to enter into this Agreement; and

It has taken all necessary corporate and other actions and obtained all relevant consents to enable it to enter into and perform its obligations under this Agreement.

Each Party undertakes that it will not knowingly infringe any intellectual property rights of any third party in conducting the Project or performing its obligations under this Agreement or creating any Foreground Intellectual Property under this Agreement, provided that neither Party shall have any obligation to conduct any investigations as to whether any third party owns any intellectual property rights.

The Parties agree that the Project involves research and development that is investigational and no warranty or representation is provided with regard to the outcome of the Project and/or the results of the Project (in particular the merchantability or fitness for a particular purpose of the results of the Project) or that the Project will result in Foreground Intellectual Property that would lead to a valid application for any application for patent, design, trade mark, copyright, or other registration available for the protection of intellectual property rights.

The Parties' covenants, representations, indemnities and warranties under this Clause 7 shall survive the expiry or termination of this Agreement.

Confidentiality

The terms of this understanding stipulate that not one or the other party should utilize nor unveil any Secret Data gotten from the other party but for the purposes of carrying out the Extend and carrying out its obligations hereunder. The accepting party concurs not to reveal any Secret Data gotten from the uncovering party to any third party but with the earlier composed assent of the uncovering party, with the exception of disclosure to its employees (referred to as Authorized Recipients) on a "need to know" basis.

The receiving Party shall ensure that Authorised Recipients are bound to and comply with the terms of this Agreement.

The obligation of confidentiality set out in this clause shall not apply to any information or materials which:

Are in the public domain;

The receiving Party can demonstrate based on written or electronic records or other recording media was already in its possession prior to its disclosure under this Agreement;

The receiving Party receives from an independent third party and the disclosure to by the independent third party to the receiving Party is not in breach of any obligation of confidentiality or non-disclosure;

Are subsequently and independently developed by the receiving Party (as evidenced by the receiving Party's written or electronic records or other recording media) who had no prior knowledge of the disclosed information; or

Are required to be disclosed by order of a court of law or appropriate government agency provided that the receiving Party informs the disclosing Party as soon as possible to enable the disclosing Party to seek a protective order or other appropriate remedy to prevent the disclosure or limit the extent of the disclosure.

Upon the termination of the Project and this Agreement, each Party shall cease use of the other Party's Confidential Information and, if so requested, shall return all copies of Confidential Information received from the other Party or shall destroy all Confidential Information received from the other Party and provide a written undertaking to the other Party that all Confidential Information has been destroyed.

Term and Termination

This Understanding might commence on the Compelling Date and might stay in constrain for the complete length of the Extend Period unless prior ended in understanding with this Clause. The Extend Period may be expanded for such assist period(s) as may be concurred from time to time by the Parties in writing. With composed take note given to the other party one month in development, either party may end this understanding at any time. If the other party is in fabric breach of any of its commitments beneath this Assentation and falls flat to cure the breach where it is able of helping inside fourteen (14) days of the non-defaulting Party's composed take

note indicating the breach and requiring its cure, either party may end this Understanding with fourteen (14) days' composed notice. If either party is in fabric breach of any of its commitments beneath this Assentation and the breach cannot be helped, either party may promptly end this Understanding with composed notice... Any expiry or termination.

Notices

All notices shall be given on writing and sent through the postal or via fax using the provided addresses and/or fax number has it is indicated in Clause 11.2 and clause 11.3 of this agreement, or to such other address or fax number as may be notified in writing by that Party for this purpose, and shall be effective notwithstanding any change of address or fax number not notified.

The respective contact particulars of the Parties are as follows:

BLOOOD SERVICE: Professor David Irving
Director, Research and Development
Australian Red Cross Blood Service
Level 3, 17 O-Riordan Street
Alexandra NSW 2000
Australia

KNBTS: Dr Josephine Githaiga,
Director, Kenya National Blood Service
KNH Grounds-Hospital Road
29804-00202
Nairobi-Kenya

The applicable fax numbers are as follows:

BLOOOD SERVICE: (61) 2 9234 2411
KNBTS: (65) 6223 8682

Relationship of the Parties

Nothing in this Assentation makes a relationship

of boss and representative, vital and operator, joint wander or organization between the Parties and no Party will hold itself out as more specialist for another.

Severability

The legitimateness, validity, and enforceability of the remaining arrangements of this Understanding stay unaffected within the occasion that any arrangement of this Understanding is found to be unlawful, invalid, or unenforceable in entire or in portion in understanding with any statute or run the show of law.

Assignment

Not one or the other Party might be entitled to dole out all or any portion of its rights, title or intrigued in or beneath this Understanding, without the earlier composed assent of the other Party.

Entire Agreement

There are no additional written or oral agreements between the Parties regarding the Project unless otherwise agreed, and the Schedule, which is a part of the Agreement and is deemed to be incorporated therein, constitutes the entire agreement and understanding of the Parties with regard to the Project. It supersedes all past communications, whether verbal or written, between the Parties, counting any past understanding or understanding shifting or amplifying the same.

Variation

No variation of this Agreement (including its Schedules and Appendices) shall be in operational but upon a written note and signature appended on by or on behalf of the parties.

Waiver

A waiver by one party of a breach by the other party of a term of this Agreement does not prevent the other party from enforcing that term in the future and does not constitute a waiver of any subsequent breach.

Force Majeure

There will be no liability on the part of either party could there arise any kind of delay or unsuccessful performance which is beyond its control, such as, floods, strikes, riots, war, fire, acts of terrorism, disease outbreak, epidemic and/or any mechanical debate. On the off chance

that such delay or disappointment proceeds past ninety (90) days, either Party will be entitled to end this agreement forthwith by a written notice.

Counterparts

Any number of counterparts to this Agreement are valid, and when taken together, they form a single legal document. Any counterpart to this agreement can be signed by either party, but until the counterparts are exchanged, this agreement has no legal effect.

Governing Law

This agreement shall be governed by the Law of South Wales, Australia if the party raising the dispute for resolution is BLOOOD SERVICE and Kenya, if the party raising the dispute for resolution is KNBTS.

Schedule 1 – Project Summary Sheet

PROJECT DETAILS	
Project 1	
Project Title	Molecular typing of blood group variants
Project Plan	☐ Tick if annexed to the Project Summary Sheet
Term of Project	
Project Start Date	[1st Nov2018]
Project Completion Date	30 June 2021
Project Summary	
Aim	The parties agree to share findings from the individual systems molecular typing, and information about any novel variants discovered, with the aim of undertaking further collaborative investigations or joint publication.
Target Milestones	

Target Milestone	Completion Date
Project reports Annually	August 2019-2021
Joint publication	October 2019
Project close out	May 2022

BLOOOD SERVICE	
Project Responsibilities	
Role	On request, the Blood Service will undertake molecular typing of blood group variants, using systems as appropriate, on provided DNA samples. Testing is subject to sufficient quantities of extracted DNA being available and will at all times be subject to the Blood Service's discretion.
Deliverables	The BLOOOD SERVICE will integrate testing with other activities with the objective of undertaking testing within four (4) weeks of receipt of a sample. A maximum of 200 samples will be accepted for testing per annum. Sharing of findings/joint publication.
Chief Investigator	Professor Robert Flower
Other Personnel	Professor David Irving Associate Professor Catherine Hyland

Contributions	
Cash Contribution	N/A (in kind contributions may include purchase and provision of kits for use at the KNBTS)
In-kind Contribution	In-kind contributions as agreed between the parties.
KNBTS	
Project Responsibilities	
Role	KNBTS will provide extracted DNA samples to the Blood Service from patients and donors, particularly those with unexpected reaction patterns.
Deliverables	Assist with joint publication.
Chief Investigator	Ms Rachel Githomi
Other Personnel	As notified
Contributions	
Cash Contribution	N/A
In-kind Contribution	In-kind contributions as agreed between the parties.

As evidenced by the date stated at the beginning, this Agreement was signed.

Australian Red Cross Society

SIGNED by
Dr Joanne Pink
For and on behalf of
Australian Red Cross Society
In the presence of

_____
Witness' signature

Witness' Name:
Address:

Kenya National Blood Transfusion Service

SIGNED by
Director,
On behalf of and for
Kenya National Blood Transfusion Service
In the presence of

_____

Witness' signature

Witness' Name:
Address:

Appendix VI: MKU ERC APPROVAL

Mount Kenya University

REF: **MKU/ERC/1884**
TO: **RACHEL NYAGUTHII GITHIOMI**

Date: 18 August 2021

REG: **PHDMLS/2018/37488**

Dear Sir/Madam,

RE: MOLECULAR CHARACTERIZATION OF RED BLOOD CELL VARIANTS (ABO, D, MNS, KELL, KIDD, DUFFY AND DANTU) IN BLOOD DONORS AT THE NATIONAL TESTING LABORATORY –KENYA BLOOD TRANSFUSION SERVICE

This is to inform you that **Mount Kenya University** has reviewed and approved your above research proposal. Your application approval number is **957.** The approval period is **18/08/2021 - 17/08/2022.**

This approval is subject to compliance with the following requirements;

i. Only approved documents including informed consents, study instruments, MTA will be used
ii. All changes including amendments, deviations and violations are submitted for review and approval by **Mount Kenya University**
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to **Mount Kenya University** within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affect the safety or welfare of study participants and others or affect the integrity of the research must be reported to **Mount Kenya University** within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal
vii. Submission of an executive summary report within 90 days upon completion of the study to **Mount Kenya University**

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://research.portal.nacosti.go.ke and also obtain other clearances needed.

Yours sincerely,

The Chairman
Mount Kenya University
Ethics Review Committee
P. O. Box 342 - 0100, Thika

Dr. Peter G. Kirira
Chairman, Mount Kenya University IERC

Appendix VII: MKU Nacosti Introductory Letter

Mount Kenya University

DIRECTORATE OF GRADUATE STUDIES

PHDMLS/2018/37488

19th August, 2021

The Director, Research Coordination Division
National Commission for Science, Technology & Innovation
Utalii House, 8th & 9th Floor
P.O Box 30623- 00100
NAIROBI

Dear Sir/Madam,

RE: RACHEL NYAGUTHII GITHIOMI - REGISTRATION NO. PHDMLS/2018/37488

The purpose of this letter is to introduce the above named student who is pursuing **Doctor of Philosophy in Medical Laboratory Sciences** in the Department of **Medical Laboratory Sciences** in **Medical School.**

The title of her research is ***"Molecular Characterization of Red Blood Cell Variants (ABO, D, MNS, Kell, Kidd, Duffy and Dantu) in Blood Donors at the National Testing Laboratory –Kenya Blood Transfusion Service."***

She has been cleared by the University's Ethics Review Committee (Certificate attached) and now has to proceed to the field to collect data for his research between **August and December, 2021.**

Any assistance accorded to her will be highly appreciated.

Thank you.

Mount Kenya University
P.O. Box 342-01000, THIKA
Office of the Director
Graduate Studies

Dr. Samuel M. Karenga, Ph.D.
Director, Graduate Studies
Enc.

Appendix VIII: National Commission for Science Technology & Innovation Approval

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 809326

Date of Issue: 06/September/2021

RESEARCH LICENSE

This is to Certify that Ms.. RACHEL Nyaguthii GITHIOMI of Mount Kenya University, has been licensed to conduct research in Nairobi on the topic: MOLECULAR CHARACTERIZATION OF RED BLOOD CELL VARIANTS (ABO, D, MNS, KELL, KIDD, DUFFY AND DANTU) IN BLOOD DONORS AT THE NATIONAL TESTING LABORATORY –KENYA BLOOD TRANSFUSION SERVICE for the period ending : 06/September/2022.

License No: NACOSTI/P/21/12583

809326

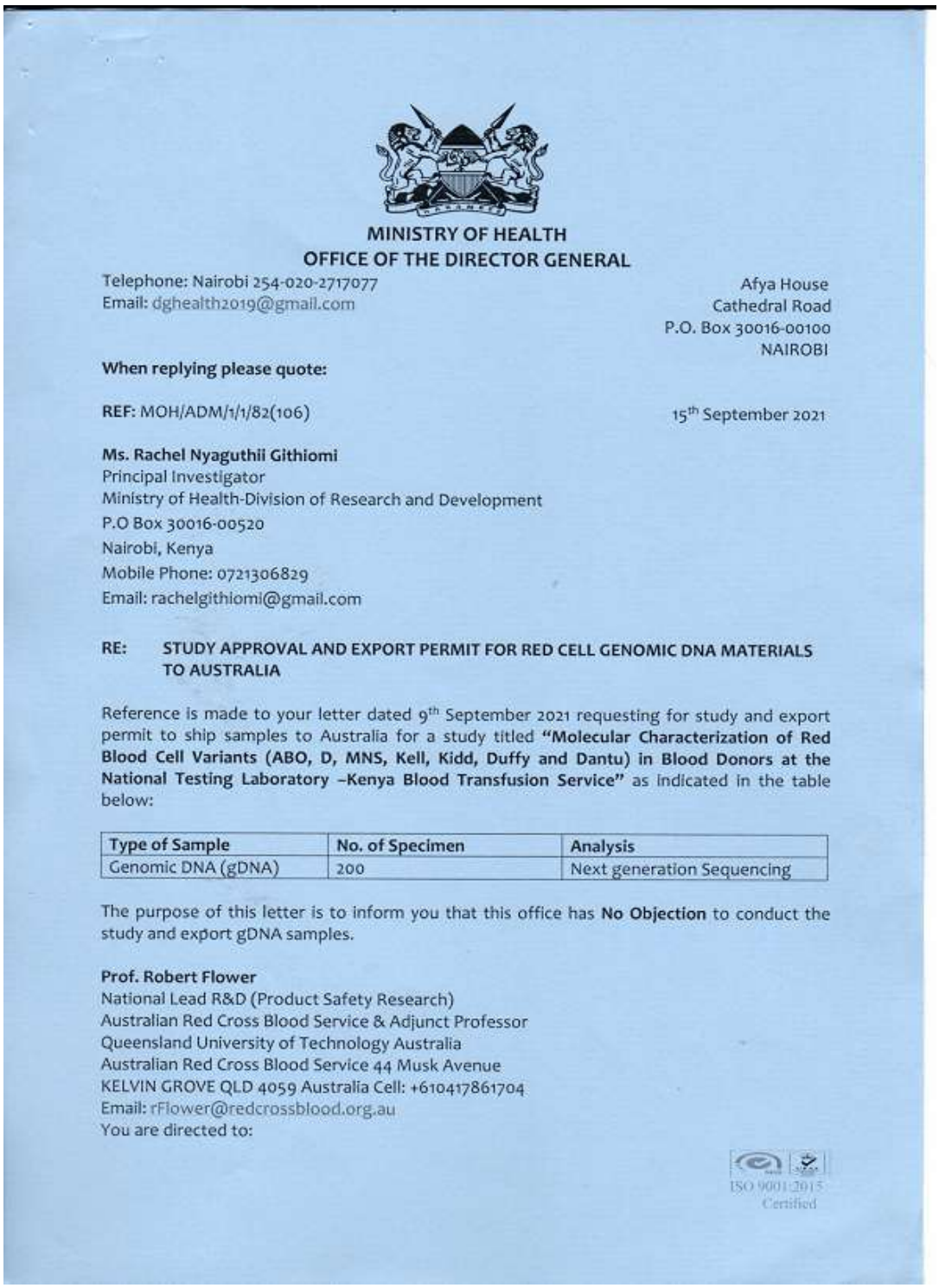
Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

Appendix IX: Ministry of Health Approval



MINISTRY OF HEALTH
OFFICE OF THE DIRECTOR GENERAL

Telephone: Nairobi 254-020-2717077
Email: dghealth2019@gmail.com

Afya House
Cathedral Road
P.O. Box 30016-00100
NAIROBI

When replying please quote:

REF: MOH/ADM/1/1/82(106)

15th September 2021

Ms. Rachel Nyaguthii Githiomi
Principal Investigator
Ministry of Health-Division of Research and Development
P.O Box 30016-00520
Nairobi, Kenya
Mobile Phone: 0721306829
Email: rachelgithiomi@gmail.com

RE: STUDY APPROVAL AND EXPORT PERMIT FOR RED CELL GENOMIC DNA MATERIALS TO AUSTRALIA

Reference is made to your letter dated 9th September 2021 requesting for study and export permit to ship samples to Australia for a study titled **"Molecular Characterization of Red Blood Cell Variants (ABO, D, MNS, Kell, Kidd, Duffy and Dantu) in Blood Donors at the National Testing Laboratory –Kenya Blood Transfusion Service"** as indicated in the table below:

Type of Sample	No. of Specimen	Analysis
Genomic DNA (gDNA)	200	Next generation Sequencing

The purpose of this letter is to inform you that this office has **No Objection** to conduct the study and export gDNA samples.

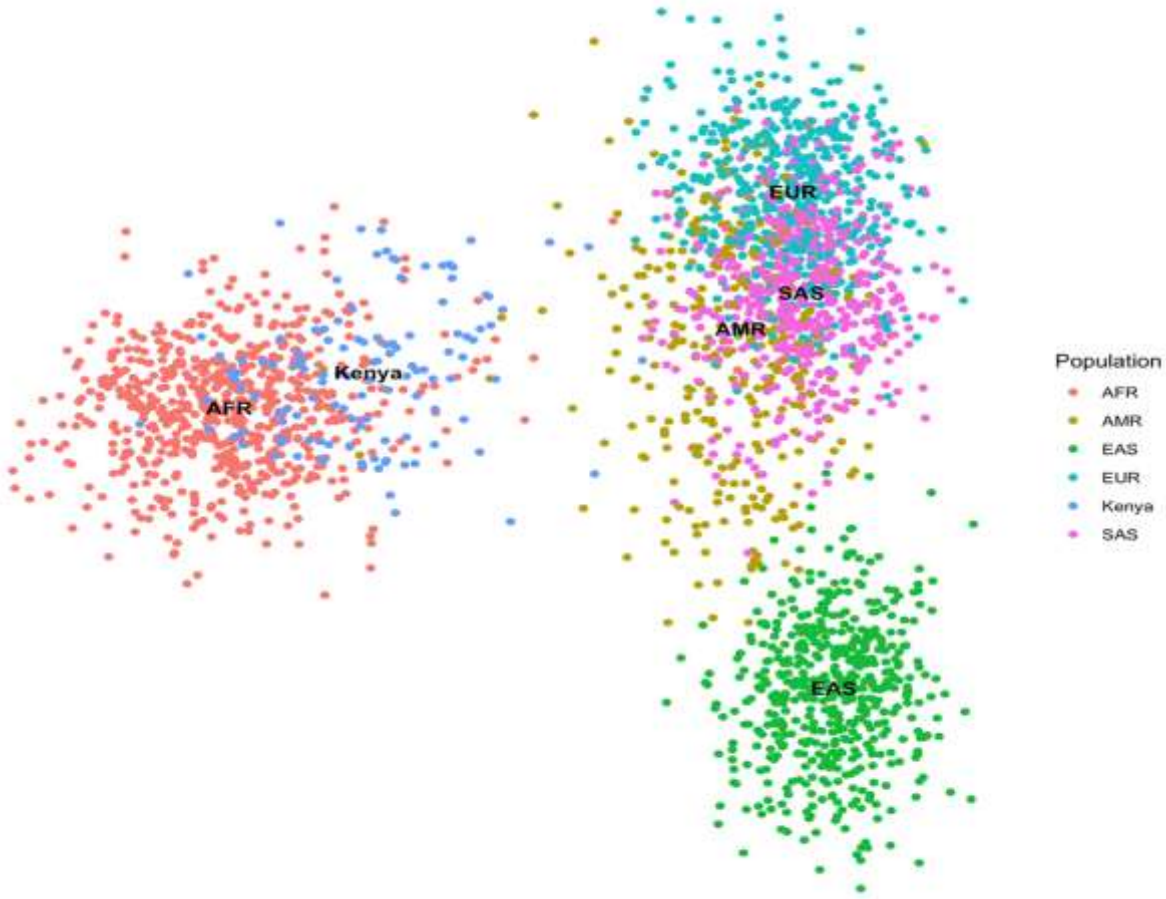
Prof. Robert Flower
National Lead R&D (Product Safety Research)
Australian Red Cross Blood Service & Adjunct Professor
Queensland University of Technology Australia
Australian Red Cross Blood Service 44 Musk Avenue
KELVIN GROVE QLD 4059 Australia Cell: +610417861704
Email: rFlower@redcrossblood.org.au
You are directed to:

ISO 9001:2015 Certified

Appendix X: Kenya Red cell data in all the Nations Narrative

Data alignment is the process of synchronization of the essential representations and descriptions of variables or elements in order to yield principles for comparing them with existing data. It is also regarded as a criterion for Data integration. Data integration has some requirements that will be considered prior to commencing the process of incorporation. For two or more datasets from different sources to be combined, the Variables, and the values of those Variables must have similarities and can be matched between both datasets. In this study, the generated genomic data was aligned with similar data of blood group phenotypes (ABO,D,C,E, MNS, KEL, JK, and FY) among other nations to include; Africa, America, Europe, East Asia Summit (Brunei, Cambodia, Indonesia, Laos, Malaysia, Myanmar, the Philippines, Singapore, Thailand, Vietnam) along with Australia, China, India, Japan, New Zealand, the Republic of Korea, Russia and the United States; Scandinavian States(SAS); Denmark, Norway and Sweden as shown in figure 6. The data alignment has shown that the phenotypes found in Kenya are also available in the nations though in varying distribution as shown in figure 6. The datasets plots of Kenya in comparison with the African nations is a proof to show that there is limited studies that have been carried out on blood group phenotypes among the countries found in the Africa continent. The plot further indicate that there is heterogeneity or distribution variations distribution of blood group phenotypes among the nations and more studies have been carried out on the same among the developed nations. This study has generated adequate evidence that can be applied to extend this type of research East Africa and other Sub-Saharan Africa countries considering their clinical significance in blood transfusion therapy. The generated evidence can also be useful in reviewing and documented testing and blood management guidelines in Kenya and Africa to safety improve on service delivery and safety.

Appendix XI: Kenya Red Cell Dataset Plots Among Nations



The red dots represent African Nation while the blue ones shows Kenya among the nations of the world (genotype data presented in this report).Alignation of Kenya Red cell data in all the nations

Appendix X1I: Similarity Report

MOLECULAR CHARACTERIZATION OF RED BLOOD CELL VARIANTS AMONG BLOOD DONORS AT THE NATIONAL TESTING LABORATORY NAIROBI –KENYA BLOOD TRANSFUSION SERVICE

ORIGINALITY REPORT

18%	14%	10%	3%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.benallahealth.org.au Internet Source	1%
2	emea.support.illumina.com Internet Source	1%
3	content.edgar-online.com Internet Source	1%
4	docksci.com Internet Source	<1%
5	www.frontiersin.org Internet Source	<1%
6	repository.mut.ac.ke:8080 Internet Source	<1%
7	onlinelibrary.wiley.com Internet Source	<1%
8	rhesusbase.info Internet Source	<1%